Effects of Sleep Modulation on Stroke Recovery in Rats

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"Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: 'Ye must have faith'. It is a quality which the scientist cannot dispense with."

Max Planck, 1932

1. SUMMARY

The general aim of the present thesis was to investigate the role of sleep in the recovery process of the brain after ischemic stroke in rats, and in particular to understand whether sleep promotion could improve post-stroke recovery.

Sleep-wake disturbances have been described in patients suffering from stroke and appear to have a negative impact on rehabilitation and long-term outcomes. In the experimental stroke model, sleep disruption has detrimental effects on infarct size, expression of plasticity-related genes, and functional recovery, while sleep stimulant gamma-hydroxybutyrate (GHB) accelerates stroke recovery in mice. Yet, the role of sleep and its underlying mechanisms in modulating the injured brain and clinical outcomes are poorly known. Promotion of neuroplasticity during recovery may represent an effective therapeutic strategy and, since sleep has been implicated in facilitation of neuroplasticity, approaches targeting sleep may lead to development of treatment able to improve long-term functional outcome after stroke.

The first goal was to evaluate whether two pharmacological agents, baclofen (Bac) and GHB would induce a physiological sleep in rats. Recently, a lot of effort have been put to create drugs acting through GABA receptors in order to promote physiological sleep for therapeutic purposes. Both drugs, which act through $GABA_B$ receptors, have been shown to promote sleep in humans, but their effects in rodents remain unclear. The results of this study demonstrated that Bac and GHB induced a non-physiological state characterised by atypical behaviour and abnormal electroencephalogram (EEG) pattern, and affected vigilance in rats. However, the principal finding was that Bac, but not GHB, had sleep-promoting properties (facilitated and consolidated sleep). Therefore, as the next step we decided to use Bac as a sleep-promoting drug and evaluate effects on functional recovery after stroke.

The second study aimed at investigating stroke outcome following repeated treatment with Bac in a rat model of focal cerebral ischemia. The first injections of Bac or saline were given 24 h after initiation of ischemia and then twice daily for 10 consecutive days. The data demonstrated that repeated Bac treatment after stroke affected sleep, neuroplasticity and motor function but not the size of the brain damage. Thus, Bac administration increased non-rapid eye movement (NREM) sleep amount and

improved motor function recovery concomitantly with enhanced axonal sprouting and neurogenesis. Furthermore, although Bac had no effect on the size of the lesion volume, it reduced atrophy of the corpus callosum. These results indicate that delayed repeated Bac treatment promotes neuronal plasticity after stroke and thereby benefits motor function recovery. We suggest that observed effects might be mediated by sleep, emphasizing the importance of sleep in recovery processes.

In summary, the results of this work contribute to the understanding of the sleep-modulating effects on stroke recovery processes, indicating that pharmacological sleep promotion could mediate and enhance endogenous recovery mechanisms in a rat model of focal cerebral ischemia. Furthermore, these studies not only advance our knowledge in basic principles of sleep function in the injured brain but may also offer a rationale for a novel strategy to promote recovery after stroke, and possibly other brain injuries, by developing sleep-modulating treatments.

2. ZUSAMMENFASSUNG

Das Ziel der vorliegenden Arbeit war es die Rolle von Schlaf in Bezug auf den Genesungsprozess des Gehirns nach einem ischämischen Schlaganfall bei der Ratte zu untersuchen. Im Fokus stand die Frage, ob Schlafförderung einen Einfluss auf eine verbesserte Genesung nach einem Schlaganfall haben könnte.

Bei Patienten nach einem Schlaganfall wurden Störungen des Schlaf-Wach-Verhaltens beschrieben. Diese scheinen negative Auswirkungen auf die Rehabilitation und das Langzeitergebnis zu haben. Im experimentellen Ansatz für Hirninfarkte führen Schlafstörungen einerseits zu nachteiligen und schädlichen Effekten in Bezug auf Infarktgrösse, Expression von Plastizitäts-relevanten Genen und die funktionelle Genesung, andererseits führt eine Schlafförderung, eingeleitet durch das Stimulanz Gamma-Hydroxybutyrat (GHB), zu einer beschleunigten Regeneration bei Mäusen. Dennoch ist die Rolle von Schlaf und den dahinter stehenden Mechanismen, welche für die Modulierung des verletzten Gehirns und das klinische Ergebnis verantwortlich sind, bislang nur ungenügend untersucht. Die Förderung der Neuroplastizität während der Genesung könnte eine effektive therapeutische Strategie darstellen. Zudem könnte ein Ansatz, mit Fokus auf den Schlaf, zu einer neuen Behandlung führen, welche einen positiven Einfluss auf das funktionelle Langzeit Ergebnis nach einem Hirnschlag hätte. Dies vor allem im Hinblick darauf, dass der positive Einfluss von Schlaf auf die Begünstigung der Neuroplastizität bereits belegt wurde.

Der erste Teil der hier vorliegenden Arbeit, war die Evaluierung zweier Medikament, Baclofen (Bac) und GHB, hinsichtlich ihres Einflusses auf die physiologische Schlafförderung bei Ratten. Kürzlich wurden Bemühungen intensiviert, Medikamente zu therapeutischen Zwecken zu untersuchen, welche über den GABA Rezeptor agieren und dadurch den physiologischen Schlaf beeinflussen. Bei beiden Medikamenten, welche über den $GABA_B$ Rezeptor wirken, wurde eine Schlafförderung beim Menschen nachgewiesen. Deren Einfluss auf Nager ist jedoch unbekannt. Die Ergebnisse der hier vorliegenden Studie haben gezeigt, dass sowohl Bac als auch GHB einen nicht-physiologischen Zustand einleiteten, welcher charakterisiert wird durch atypisches Verhalten, abnormale Elektroencephalogram (EEG) Aktivität und einen Einfluss auf die Wachsamkeit der Ratten. Zusätzlich aber zeigten die Resultate die

schlaffördernde Wirksamkeit (erleichternd und konsolidierend) von Bac jedoch nicht von GHB. Daher wurde Bac als schlaffördernde Mittel zur Evaluierung der Effekte auf die funktionelle Genesung nach einem Schlaganfall gewählt.

Der zweite Teil dieser Arbeit befasst sich mit der Untersuchung des Einflusses der wiederholten Gabe von Bac auf den Ausgang einer fokalen zerebralen Ischämie im Rattenmodell. Erste Injektionen von Bac oder Saline erfolgten 24 Stunden nach Initiation der Ischämie und jeweils zwei Mal täglich an den darauffolgenden 10 Tagen. Die Daten ergaben, dass eine Bac Behandlung den Schlaf, die Neuroplastizität und auch die motorischen Funktionen beeinflusst, jedoch keinen Einfluss auf die Grösse der geschädigten Hirnregion hat. Daraus lässt sich schliessen, dass die Bac Gabe eine Zunahme des Non-Rapid Eye Movement (NREM) Schlafes als auch eine verbesserte Regeneration der motorischen Funktionen nach sich zieht. Diese wurden von einem verstärkten axonalem Sprouting und Neurogenese begleitet. Die Resultate zeigten ebenso, dass obwohl Bac keinen Effekt auf die Grösse des Läsionsvolumens hatte, es die Atrophie im Corpus callosum reduzierte. Diese Daten indizieren, dass eine verzögerte und wiederholte Behandlung mit Bac die neuronale Plastizität nach einem Schlaganfall fördert und dadurch zu einer verbesserten Regeneration der motorischen Funktionen führt. Wir vermuten, dass die beobachteten Effekte durch Schlaf vermittelt werden und unterstreichen dadurch die Wichtigkeit des Schlafes im Regenerationsprozess.

Zusammenfassend tragen die Ergebnisse dieser Arbeit zum Verständnis der Schlaf-modulierenden Effekte auf die Regenerationsprozesse nach einem Schlaganfall bei. Sie weisen darauf hin, dass pharmakologische Schlafbeeinflussung endogene Regenerationsmechanismen im Rattenmodell der fokalen zerebralen Ischämie auslösen oder verstärken kann. Des Weiteren, führen die in dieser Arbeit vorgestellten Ergebnisse nicht nur zum näheren Verständnis der grundlegenden Mechanismen der Schlaffunktion im geschädigten Gehirn, sondern bieten auch ein Prinzip für eine neuartige Strategie zur Förderung der Genesung nach einem Schlaganfall und, möglicherweise, auch anderer Gehirnverletzungen, durch die Entwicklung neuer Schlaf-modulierender Behandlungen.

3. INTRODUCTION

3.1 Stroke

Basic pathophysiology and current treatments

"If one desired to guarantee the failure of a drug in clinical trials then one should develop a compound for the treatment of stroke". Although that comment was made in early 1980s by a notable neurosurgeon, it continues to be held by many clinicians and people in pharmaceutical industry. The reason for this ongoing pessimism is that, beside thrombolysis, there are still no drugs which could be given to stroke patients soon after the onset of symptoms to minimize the subsequent neurological problems and to improve functional recovery.

Stroke still remains the leading cause of chronic adult disability and the third leading cause of death in major industrialized countries, behind heart disease and cancer (Duncan, 2002, Burns et al., 2009). There are two major types of stroke: ischemic and hemorrhagic. The most frequent is ischemic stroke, which results from an occlusion by thrombus or embolus of a cerebral artery. This leads to a reduction in the brain blood flow, and to a major decrase in the supply of oxygen and glucose in the affected regions. Neurons that are deprived of their normal metabolic substrates cease to function in seconds and show signs of structural damage within few minutes (Dirnagl et al., 1999). Neuronal death consequently leads to damage in functional networks and to impairment of sensation, movement or cognition. Ischemic strokes are often associated with disorders that involve clotting of the blood or arterial inflammation. Hemorrhagic stroke is instead caused by intracerebral bleeding (within the brain tissue) or subarachnoid hemorrhage. Hemorrhagic stroke is usually linked to disorders, which prevent or reduce normal blood clotting, and which therefore result in an increased potential for bleeding. Moreover, it could be associated with blood vessels malformations, such as weakness, causing a bulge of the walls known as aneurysms. Even though approximately 75% people survive their initial stroke, just one third of them recover completely. According to the World Health Organization (Organization., 2007) 15 million people suffer stroke worldwide each year. Of these, five million die and another five million remain permanently disabled. This long-term disability implies that the majority of the costs

related to stroke is consumed on the support of stroke survivors, the number of whom is expected to keep growing due to the natural aging of the population. For these reasons stroke is, and will continue to be one of the most challenging neurological diseases. Therefore, substantial advances in prevention and treatment of stroke are of principal importance.

Despite increasing knowledge about mechanisms occurring in the brain after ischemic injury effective treatments available for stroke patients are still lacking. So far, the only therapy that has been approved for clinical use is the administration of recombinant tissue plasminogen activator (rt-PA) which by lysing embolus or thrombus, restores the blood flow in the compromised brain region and therefore protects damaged ischemic cells from death. The area of the most drastic blood flow reduction (the ischemic core) is surrounded by the area of potentially salvageable tissue and partially preserved metabolism. This area, called ischemic penumbra, is compromised by the low blood flow, but can be protected either by reflow or by administration of neuroprotectants. Although thrombolysis is clinically effective in treating acute ischemic stroke (1995), its utilization is currently very low and limited by several factors. Because of the narrow therapeutic time window (rt-PA must be given within 4.5 h of symptoms onset), it can be used to treat a very small proportion (less than 10%) of the stroke victims evaluated in the emergency room. Moreover, not all of the patients qualify for and benefit from thrombolysis (1995, Hacke et al., 2008, Ahmed et al., 2010), as it might increase the risk of symptomatic intracranial hemorrhage (Zhang et al., 2012). The second major approach in the research of treatment for ischemic stroke is enhancing neuroprotection in order to salvage the brain tissue in the penumbra. A possible way to achieve that is by the use of drugs that interfere with the mechanisms of the 'ischemic cascade' and block the pathological processes, minimizing the subsequent neurodegeneration (Dirnagl et al., 1999). Without such an intervention the cells in the penumbra will die and the ischemic core will expand (Fig. 3.1.1). Clinical trials evaluating neuroprotective drugs for stroke were initiated in the 1980s and are still in the progress (Auriel and Bornstein, 2010). However, after decades of research and a multitude of neuroprotective compounds, which were promising in animal models of stroke, no drug was proven beneficial to stroke recovery in the clinical trial phase (Green and Shuaib, 2006, Ginsberg, 2009, Auriel and Bornstein, 2010). These dissapointing results suggest that, in stroke patients, improving neuroprotection might

not be as straightforward as it is in the experimental models. This raised an uncertainty about this type of therapeutic strategy alone in treating stroke patients and reducing neurological deficits. Therefore, development of new approaches for ischemic stroke therapies has become crucial.

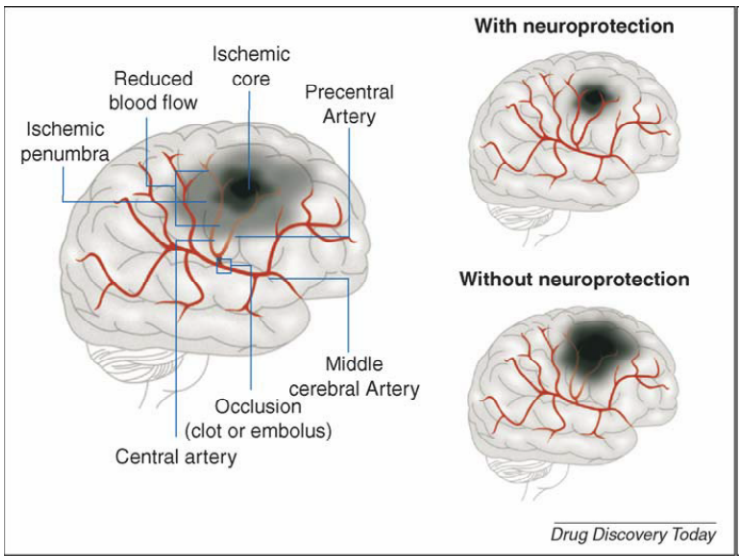

Fig. 3.1.1 An occlusion of a branch of the middle cerebral artery.
The middle cerebral artery has an indication of the ischemic core area and the penumbra. The figure shows the spread of damage as occurs with and without neuroprotective drug administration (redrawn from (Green and Shuaib, 2006)).

In search of new treatments, numerous neurorestorative therapies such as transcranial magnetical stimulation (TMS) (Talelli and Rothwell, 2006, Hallett, 2007), transcranial direct current stimulation (tDCS) (Hummel and Cohen, 2006), cell-based and pharmocological therapies (Zhang and Chopp, 2013) are currently being investigated. The main approach of restorative therapies is to support and stimulate brain repair processes during the subacute and chronic phases after insult, with the aim of improving functional recovery (Cramer, 2008, Zhang and Chopp, 2013).

"Since regeneration of transectioned central axons has never been convincingly demonstrated in higher mammals, it seems in most instances that one must resort to the assumption that intact fibers take over for the damaged ones." (Brodal, 1973). This statement written in 1973 by Alf Brodal, based on his own experience after stroke, has appeared to be indeed the case. Although brain damage after ischemic injury can be devastating, a certain spontaneous degree of restoration of sensorimotor functions occurs both in humans and animal models (Murphy and Corbett, 2009, Johansson,

2011). Loss of function attributable to stroke is partly due to neuronal death in the infarcted tissue but it is also a result of cell dysfunction in the areas surrounding the infarct. These areas encompass the part of the underperfused penumbra, the non-ischemic peri-infarct tissue and remote (including contralateral) brain areas that are connected to the area of tissue damage (Wieloch and Nikolich, 2006). Initially, the neurological deficits reflect injury to the core as well as the penumbra (Fig. 3.1.2). As collateral perfusion develops, brain function can be restored within the penumbra (Furlan et al., 1996). However, the structural lesion consolidates over time and might recruit parts of the ischemic penumbra into infarction. Thus, symptoms can regress while the lesion actually expands. After days to weeks, neurological deficits reflect the size and location of the structural lesion more closely. Recovery of function from this time point is best explained by plasticity and tissue reorganization (Dirnagl et al., 1999). Accordingly, the spontaneous functional recovery has been attributed to brain plasticity which by reorganizing the undamaged neuronal circuits undertakes the functionality of the circuits damaged by stroke. Recovery processes, both in humans and animal models, primarily engage ipsilateral brain regions, although if the damage is severe, contralateral brain areas are also recruited (Biernaskie et al., 2005, Kim et al., 2005, Ward, 2005).

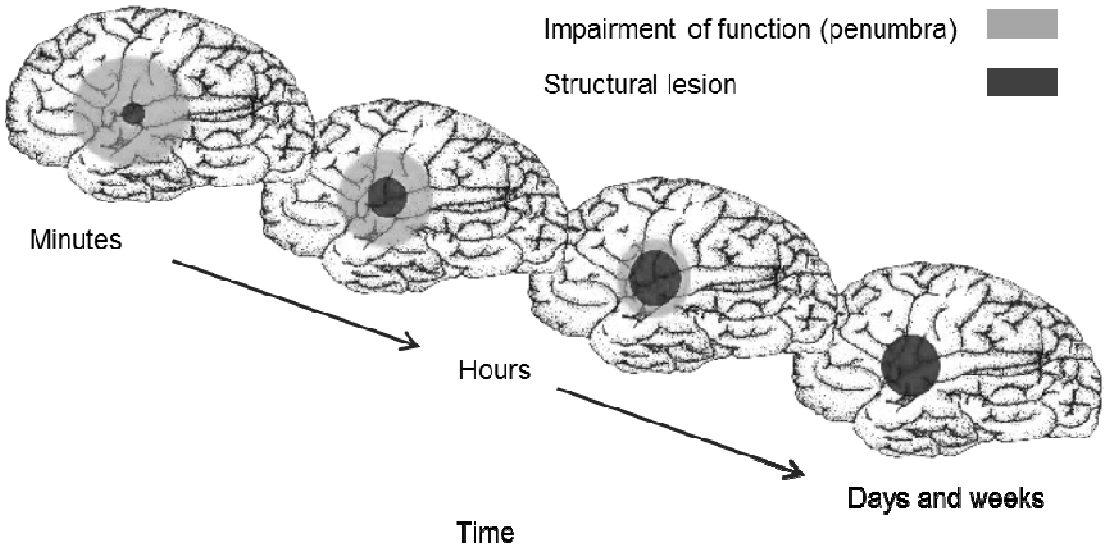

Figure 3.1.2 Regression of the functional neurological deficit while the structural lesion grows.
Early in the course of stroke, clinical symptoms mostly reflect an impairment of function (light grey) but not necessarily a structural lesion (dark grey). Over time, some areas either spontaneously, or because of therapy, recover function, which explains why symptoms in patients can regress while the structural lesion actually grows (adapted from (Dirnagl et al., 1999)).

Stroke and brain plasticity

The term plasticity was introduced in neurosciences by an Italian psychiatrist Ernesto Lugano in 1906 (Berlucchi, 2002), however the important studies in this field were initiated by Donald Hebb. More than half a century ago, he postulated that neuronal cortical connections are strengthened and can be remodeled by our experience (Hebb, 1947). Since then, many studies have demonstrated chemical and anatomical plasticity in the cerebral cortex of adult animals (Bennett et al., 1964, Rosenzweig, 1966). Another aspect of brain plasticity, first and most extensively demonstrated by Merzenich and coworkers, was that cortical representation areas and cortical maps can be modified by sensory input, experience, and learning, as well as in response to brain lesions (Merzenich et al., 1983, Merzenich et al., 1984, Jenkins and Merzenich, 1987, Xerri et al., 1998). Thus, the potential relevance of those data for stroke rehabilitation was proposed more than a decade ago (Jenkins and Merzenich, 1987). Furthermore, synaptic plasticity and dendritic spines number in cortical horizontal connections has been proposed to underlie cortical map reorganization (Hess and Donoghue, 1994, Buonomano and Merzenich, 1998). At the structural level, neuronal plasticity can be defined in terms of dendritic and axonal arborization, number of neurons, spine density, synapse number and size, and receptor density (Pekna et al., 2012). These structural components of neuronal plasticity jointly determine the complexity of neuronal networks and their activity, and contribute to functional recovery after stroke and other central nervous system injuries (Pekna et al., 2012). Numerous mechanisms are likely to be involved in brain plasticity. Modifications of synaptic connection and reorganization of adult cortical areas are thought to involve long-term potentiation (LTP) and long-term depression (LTD). LTP leads to an increased postsynaptic response and therefore increases synaptic strength, whereas LTD is characterised by a reduction in synaptic strength. Both LTP and LTD are important mechanisms by which information is thought to be stored in the mammalian central nervous system (Bear and Malenka, 1994, Feldman et al., 1999). However, despite 30 years of research in this area the detailed mechanisms remain not fully understood. Brain plasticity occurs at many levels, from molecules to cortical reorganization, therefore strategies promoting synapse and network level plasticity leading to the recovery of function, appear to be very promising.

The stroke outcome may be influenced by several factors: the time after injury, lesion site and the integrity of cortical and subcortical connections. Thus, the location

and sequence of post-stroke mechanisms are crucial in the recovery process and can offer different types of treatment opportunities. The recovery of neurological function in patients who have suffered a stroke can be mimicked in experimental animal models, in which a repertoire of overlapping recovery-promoting processes have been identified (Fig. 3.1.3). After stroke, the central nervous system (CNS) rearranges its neural networks to rapidly reorganize structure and function. Recovery process involves distinct phases, i.e. activation of cell repair, functional cell plasticity (changing the properties of existing neuronal pathways) and neuroanatomical plasticity leading to the formation of new connections. After the initial events during the first days of recovery, the synapses of surviving neurons in addition to silent synapses and pathways of areas remote from the infarct, are activated. Early during recovery, new glial cells and neuroblasts are generated and migrate into peri-infarct areas or contralateral remote regions. During the following 2–3 weeks, neuroanatomical plasticity (axonal sprouting, dendritic and spine growth) is enhanced by the milieu created by surviving neurons and proliferating parenchymal cells. In addition, angiogenesis is stimulated, and contributes to the formation of new vessels in the peri-infarct areas. Finally, once novel neural networks are established, they are stabilized by an experience driven learning process (Wieloch and Nikolich, 2006). Because the spontaneous recovery is usually limited and therefore insufficient, the strategy of restorative therapies is to promote the endogenous brain restorative events in order to boost brain remodeling and reorganization (Nudo et al., 1996, Traversa et al., 1997). These targeted endogenous processes underlying brain plasticity include stroke-induced change in expression of neuroplasticity-related markers (Carmichael et al., 2005), neurogenesis (Zhang et al., 2001a, Hou et al., 2008), synaptogenesis (Stroemer et al., 1998), angiogenesis (Chen et al., 2003), axonal sprouting (Papadopoulos et al., 2002, Wiessner et al., 2003), inhibition of astrogliosis (Li et al., 2005b), anti-inflammation (Bacigaluppi et al., 2009). Indeed, during the recent years this concept has received support in a number of animal studies showing that application of pharmacological agents and various types of cells (including stem cells and neuronal precursors) can improve functional recovery after stroke (Shyu et al., 2004, Chopp et al., 2009, Zhang and Chopp, 2013). Yet, the success of any given therapeutic intervention depends on how well it interacts with the new, stroke-induced functional network. At present, only few restorative therapies could be translated into clinical trials. Thus, methods inducing or boosting neuronal plasticity in the affected

brain combined with currently available acute treatments may represent a new effective therapeutic strategy for stroke therapy. The hope is that by understanding mechanisms which lead to functional recovery we could enhance the naturally occurring recovery capabilities of the brain.

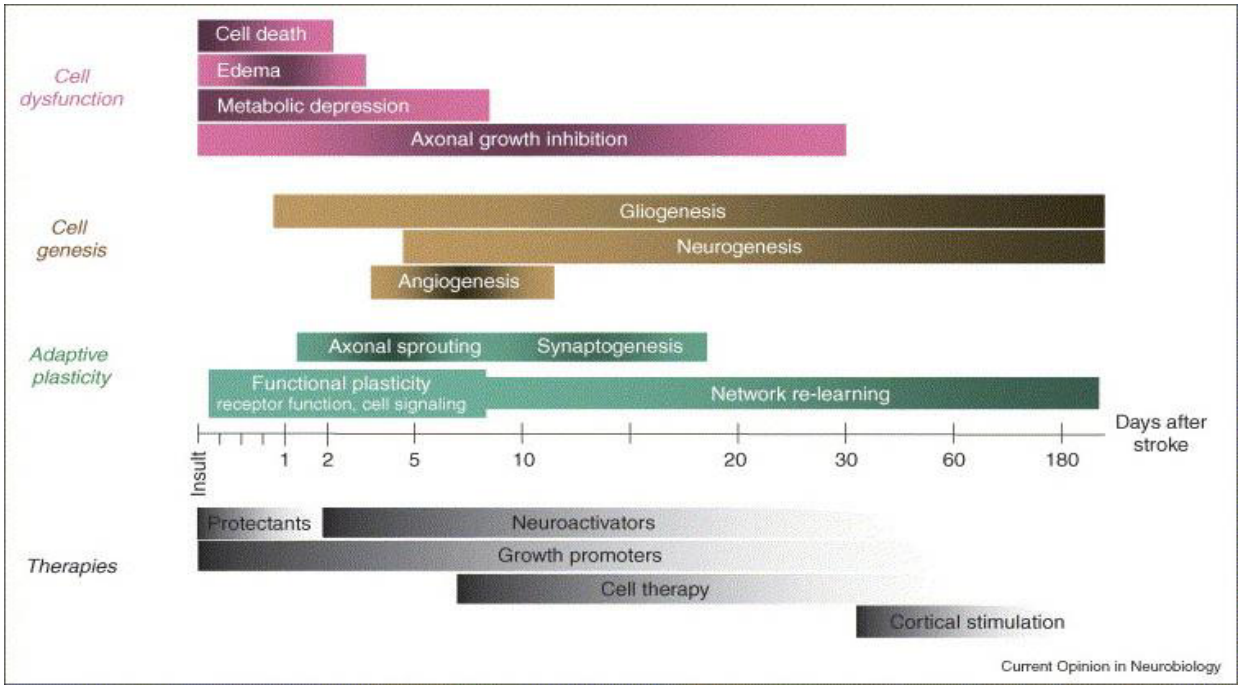

Figure 3.1.3 An overview of the activated parallel processes and therapeutic approaches following stroke.

The temporal sequence of events is shown along a semi-logarithmic schematic timeline of 180 days after injury. Darker shading highlights the maximum intensity of the specific mechanism. Processes that are detrimental towards recovery are shown in pink. Processes of cell genesis are shown in brown, whereas those that underlie adaptive plasticity are shown in green. Prospective therapies that focus on neuroprotection and enhancement of regeneration and functional recovery are shown in gray (redrawn from (Wieloch and Nikolich, 2006)).

3.2 Sleep

Physiology of sleep

Sleep, a phylogenetically, highly preserved process identified in all the species studied so far, is particularly well developed in the human brain (Cirelli and Tononi, 2008). It is a natural, periodically recurring state of inactivity, characterised by the loss of consciousness, immobility, typical sleeping posture and reduced responsiveness to the external stimuli. In contrast to humans exhibiting a single sleep period of approximately 8 hours during the dark phase, rodents have a polyphasic sleep pattern with sleep episodes throughout the 24 hours, dominating during the light period (Tobler, 1995). In addition to sleep-specific behaviour, a reliable definition of vigilance states can be obtained by measuring characteristic changes in the brain electrical activity by electroencephalogram (EEG) and in muscle tone by electromyogram (EMG).

Based on the EEG and EMG, the same three vigilance states can be discriminated in humans and rodents: wakefulness, non-rapid eye movement (NREM) sleep or slow wave sleep (SWS) and paradoxical or rapid eye movement (REM) sleep (Fig. 3.2.1). Exclusively in humans, NREM sleep is further subdivided into stages 1 to 3. Wakefulness is characterised by the presence of low-amplitude, fast-frequency, irregular EEG pattern (beta: 14-30 Hz and gamma: 30-50 Hz frequency ranges), combined with a prominent muscle activity in the EMG. During NREM sleep the frequency of EEG activity decreases and the amplitude increases, concomitantly with decrease in the muscle tone. The main characteristic of NREM sleep is the appearance of slow wave activity (SWA; 0.5-4 Hz). REM sleep is characterised by low-amplitude, desynchronized EEG activity and complete loss of muscle tone (i.e. atonia). In rodents, the EEG is typically dominated by frequencies in the theta band (4-8 Hz) during REM sleep and phasic activity appears as twitching of the vibrissae as well as eye movements. Sleep stages alternate in the course of the night in a regular manner. The duration of the sleep cycle as well as the percentage of NREM and REM sleep during 24 h varies among species (Zepelin et al., 2005).

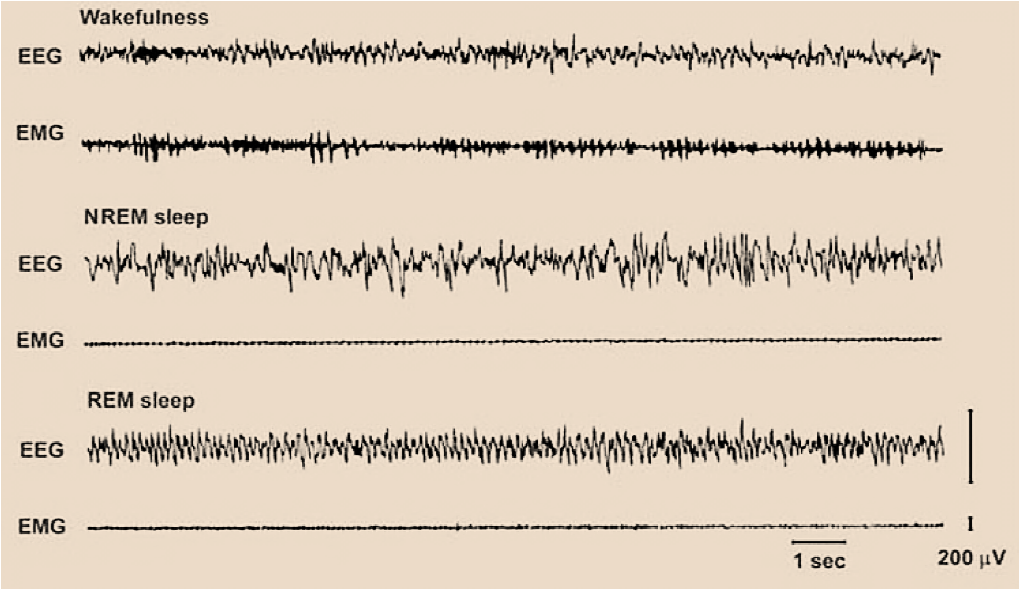

Figure 3.2.1 Electroencephalogram (EEG) and electromyogram (EMG) traces showing three vigilance states: wakefulness, NREM sleep and REM sleep, in rodents.

Sleep is regulated by three distinct mechanisms: a homeostatic, a circadian and an ultradian process. The ultradian process is represented by the alterations of NREM and REM sleep within the sleep cycle. Sleep homeostasis is represented by "process S", which increases during waking and declines during sleep. The circadian process ("process C"), on the contrary, is independent of the prior sleep-wake history and maintains an approximate 24 h rhythm through an intrinsic pacemaker. The probability to fall asleep, as well as the structure and sleep propensity, are determined by the interaction of processes S and C, as described in "The Two-Process Model" of sleep regulation (Borbely, 1982). The model explains that the longer animals stay awake, the more intensely and the longer they sleep, as measured in increased number and amplitude of slow waves, higher arousal threshold, and/or fewer awakenings.

Sleep-related mechanisms of neuroplasticity

Although the core function of sleep is still under debate, an increasing body of evidence has suggested that sleep plays an important role in neuronal plasticity at different levels of brain organization (Huber et al., 2004, Vyazovskiy et al., 2008a, Vassalli and Dijk, 2009, Bushey et al., 2011). Experimental data, as well as personal experiences, endorses that sleep after learning facilitates cognition and memory consolidation for declarative and nondeclarative tasks (Gais et al., 2000, Gais and Born, 2004, Walker and Stickgold, 2004, Stickgold, 2005). On the other hand, sleep deprivation (SD) impairs new learning

(Yoo et al., 2007) and compromises induction and/or maintenance of LTP (Davis et al., 2003, McDermott et al., 2003). Furthermore, it has been shown that expression of genes and proteins involved in plasticity processes is differentially regulated during sleep, wakefulness and following SD (Cirelli et al., 2004, Vyazovskiy et al., 2008a). Numerous genes responsible for the biosynthesis of macromolecules, especially those involved in the memebrane/myelin structure and in lipid metabolism, have been reported to be upregulated during sleep (Cirelli et al., 2004, Mackiewicz et al., 2007). This sleep-dependent regulation of gene expression is particularly relevant during the recovery from brain injury, as axonal sprouting is an important component of restorative processes. It has been also proposed that sleep may promote endogenous neurogenesis in the adult brain. Rodents subjected to SD or sleep restriction were shown to have a reduction in the rate of cell proliferation and in the number of newborn cells containing matured neuronal marker (Guzman-Marin et al., 2005, Hairston et al., 2005, Guzman-Marin et al., 2007, Meerlo et al., 2009). Functionally, the SD-induced suppression of neurogenesis has been related to the impaired learning performance after sleep disruption (Hairston et al., 2005).

Recently, a great amount of sleep research has been focused on a theory proposing that sleep is essential for synaptic homeostasis, that is the recalibration of the net synaptic weight (strength and number) to an energetically sustainable level (Cirelli et al., 2004). The proposed hypothesis, known also as the "Synaptic Homeostasis Hypothesis" (Tononi and Cirelli, 2003, 2006), assumes that uptake of information and activity during wakefulness is associated with synaptic potentiation, i.e. an upscaling of synaptic strength, while sleep, in particular NREM sleep, is associated with a global downscaling of synaptic strength. The maintenance of synapses is expensive in terms of space, energy and cellular supplies. Therefore, the synaptic downscaling during sleep is crucial to counter synaptic potentiation brought up by waking through removing weak synapses, and thereby increasing the signal-to-noise ratio in neural circuits. Otherwise the available resources of energy and space would be exceeded, which in turn could lead to an unrestrained synaptic weight growth and saturation of ability to learn (Vassalli and Dijk, 2009). This further supports the importance of sleep, which by providing optimal conditions for unbiased offline processing, is needed for renewal of brain energy stores.

SWA is a well established marker of sleep intensity, which increases as a function of prior wakefulness and declines during sleep (Borbely, 1982), as mentioned above. In the model of synaptic homeostasis theory, SWA high at the sleep onset reflects the increased overall strength of connections in the synaptic network acquired during waking period. But at the same time, slow waves represent a mechanism for downscaling which results in its gradual decrease of activity across sleep (Tononi and Cirelli, 2003, 2006). This hypothesis is supported by many studies pointing to close relationship between slow waves during NREM sleep and synaptic plasticity processes. Experimental data showed that molecular (GluR1-containing AMPA receptor [AMPAR]) and electrophysiological markers of synaptic potentiation increased after a periods of waking and decreased after recovery sleep, whereas markers of synaptic depression were found to be elevated after sleep (Lubenov and Siapas, 2008, Vyazovskiy et al., 2008a). Moreover, neuronal firing rate was increased after sustained wakefulness and decreased after sustained sleep (Vyazovskiy et al., 2009). Furthermore, several reports provided evidence for a close association between synaptic potentiation related to wakefulness and an increase in SWA during subsequent sleep. Both in humans and animals the brain regions that have been stimulated by intense activities during waking showed higher SWA in subsequent NREM sleep (Kattler et al., 1994, Huber et al., 2004, Hanlon et al., 2009), while areas that have been less engaged showed reduced SWA (Huber et al., 2006). In addition, those specific waking activities triggered induction of plasticity-related genes in the cortex and this was correlated to the extent of SWA response during the subsequent sleep episode. Observed local increase of SWA was positively correlated with the improvement in performance on the next morning (Huber et al., 2004, Huber et al., 2007, Hanlon et al., 2009).

Synaptic homeostasis concept stimulated a unique surge of research targeting the role of sleep in brain plasticity. Recently published studies challenged the theory that NREM sleep is alone responsible for establishing synaptic homeostasis. Accordingly, Cheauvette et al. (Chauvette et al., 2012) showed that during NREM sleep, the brain responsiveness was rather upscaled than downscaled, as measured by evoked potentials, while Grosmark et al. (Grosmark et al., 2012) found that downscaling in hippocampal networks might be mediated through REM sleep rather than SWS. Together, these studies speculated that NREM sleep, through slow waves, supports synaptic upscaling, while subsequent REM sleep, through theta activity, supports

downscaling. This implies that processes occurring during REM sleep should be taken into consideration while talking about synaptic plasticity. Beyond this, recent data suggests that the key mechanism launching the consolidation of newly acquired memories is the ripple-associated replay during SWS (Diekelmann and Born, 2010). It has been speculated that the interactions between ripples, i.e. high-frequency hippocampal oscillations (100-300 Hz) and slow oscillations may be crucial for synaptic consolidation. Therefore, it is clear that a number of details in connection with synaptic homeostasis should be still clarified.

Taken together, interaction between changes in sleep EEG and the plasticity-related markers at the molecular, cellular and network levels provide a link between sleep and brain plasticity.

3.3 The role of sleep in stroke recovery

The role of sleep in stroke patients

Sleep-wake disturbances are frequent after stroke and appear to have a detrimental impact on rehabilitation and long-term neurological outcome (Bassetti and Hermann, 2011). Sleep-wake disturbances observed in stroke patients include insomnia, sleep fragmentation, excessive daytime sleepiness (EDS) and, even if less frequently, hypersomnia (Hermann and Bassetti, 2009). Although there is no distinct relation to the infarct topography and size, it appears that hypersomnia and EDS are often associated with severe hemispheric stroke and damage in the paramedian thalamus, wheareas insomnia is increased with mild superficial hemispherical infarction (Autret et al., 2001, Muller et al., 2002, Vock et al., 2002, Hermann et al., 2008, Siccoli et al., 2008). Clinical outcome studies indicate that stroke patients with sleep-wake disturbances suffer more frequently from neuropsychiatric (depression and anxiety) and cognitive (memory and attention) disorders, dementia, and physical impairments (Leppavuori et al., 2002, Hermann and Bassetti, 2009). Recently, Sicolli et al. (Siccoli et al., 2008) have reported a positive correlation between the amount of sleep and cognitive functions during both the acute and the recovery phases after stroke.

Although the role of sleep in the recovery process after brain injuries remains unclear, clinical observations together with few findings in basic research suggest that

sleep may be a key modulator of stroke recovery. Accordingly, if poor functional outcome after stroke is linked to sleep-wake disturbances and sleep is instead believed to be important for the endogenous neuronal remodeling process (Huber et al., 2004, Huber et al., 2007, Hanlon et al., 2009), strategies aiming to improve and promote sleep hold a great promise for benefiting the long-term functional outcome after stroke. However, until now only few studies have investigated the relationship between these two processes and the impact of sleep on stroke pathophysiology and recovery-related brain repairing mechanisms.

Effects of sleep modulation in animal models of stroke

Experiments using animal stroke models showed that SD performed prior to global or focal cerebral ischemia attenuated stroke severity, inflammatory response and cell death in mice and rats (Moldovan et al., 2010, Cam et al., 2013). Furthermore, Gao et al. have recently carried out several studies, in which sleep has been altered either by sleep deprivation or by clinical relevant sleep-promoting drug. The main common finding of these experiments was that manipulation of sleep after focal ischemia affected stroke outcome. Thus, SD and sleep disturbances (SDis) performed shortly after stroke resulted in an aggravation of the infarct size and increase in the number of damaged cells (Gao et al., 2010). In a further study investigating the long-term outcome after stroke, disruption of sleep impeded functional recovery, as assessed by the single pellet reaching (SPR) test during a 5-week period (Fig. 3.3.1). This effect was accompanied by the impairment of the brain repairing system, such as suppressed axonal sprouting, synaptogenesis (Fig. 3.3.2A), and neurogenesis (Fig. 3.3.2B) (Zunzunegui et al., 2011). The effect of sleep disruption on expression of neuroplasticity-related genes has been also tested by probing a small set of genes that may influence axonal sprouting, such as promoting (GAP43 and c-jun), and inhibiting (neurocan, ephrin A5 and ephrin B1) genes (Carmichael, 2005). The data showed that SDis induced a substantial increase in expression of the axonal growth-inhibiting gene neurocan (Gao et al., 2010), providing evidence for sleep modulation of stroke at the molecular level. On the other hand, administration of gamma-hydroxybutyric acid (GHB), considered as a sleep-promoting drug, immediately after reperfusion accelerated motor function recovery in mice (Gao et al., 2008). However, whether these GHB-induced changes on stroke outcome were sleep-dependent has not been analysed.

Overall, these results suggest that the sleep effects on stroke can be partially similar to those occurring in the healthy brain, including the altered expression of plasticity-related genes. However, the underlying mechanisms have not been elucidated yet. Ischemic stroke, due to a sudden reduction of blood flow, triggers a cascade of events, i.e. energy failure, excitotoxicity, free radical generation and inflammation, which consequently leads to necrosis and apoptosis (Dirnagl et al., 1999). In healthy brain, sleep disruption has been reported to increase the expression of excitatory neurotransmitter receptors (Vyazovskiy et al., 2008b), to decrease the antioxidative stress parameters (D'Almeida et al., 1998, Singh et al., 2008), and to elevate the production of certain proinflammatory cytokines, such as IL-1β, IL-6, IL-17 and TNFα (Shearer et al., 2001, Vgontzas et al., 2004, van Leeuwen et al., 2009). These sleep loss-triggered changes could likely be implicated in the stroke pathophysiology via exacerbating ischemic cascade events.

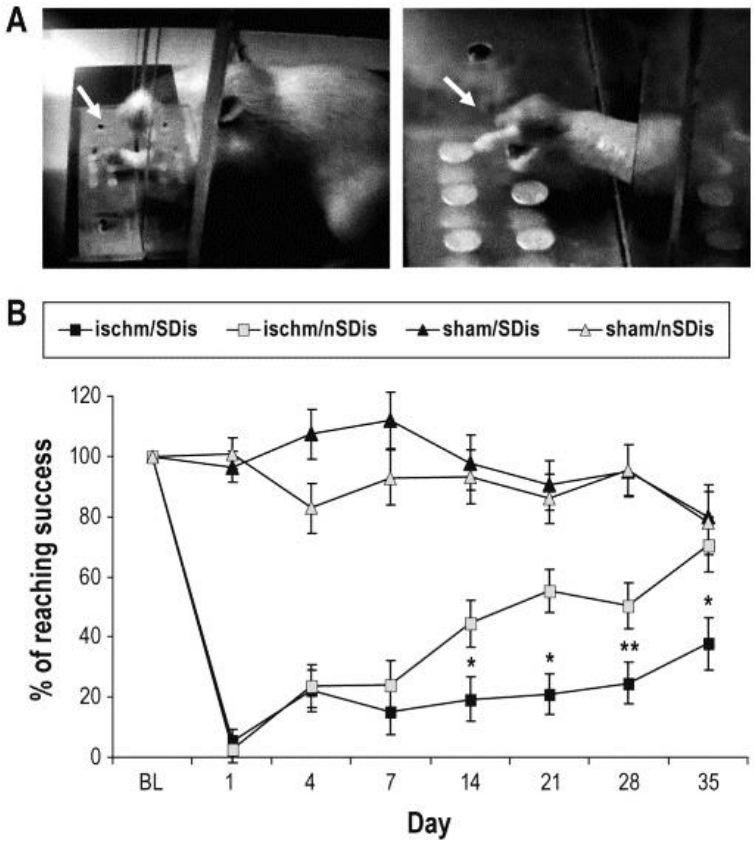

Figure 3.3.1 SDis-induced impairment in stroke recovery, evaluated by the single pellet reaching task.

A. Photographs of a rat reaching (arrows) a pellet located in an indentation on the shelf, through a slot opened in the front of a training box **B.** Reaching success is presented as percentage of the baseline (BL) *P<0.05,**P<0.01 (redrawn from (Zunzunegui et al., 2011)).

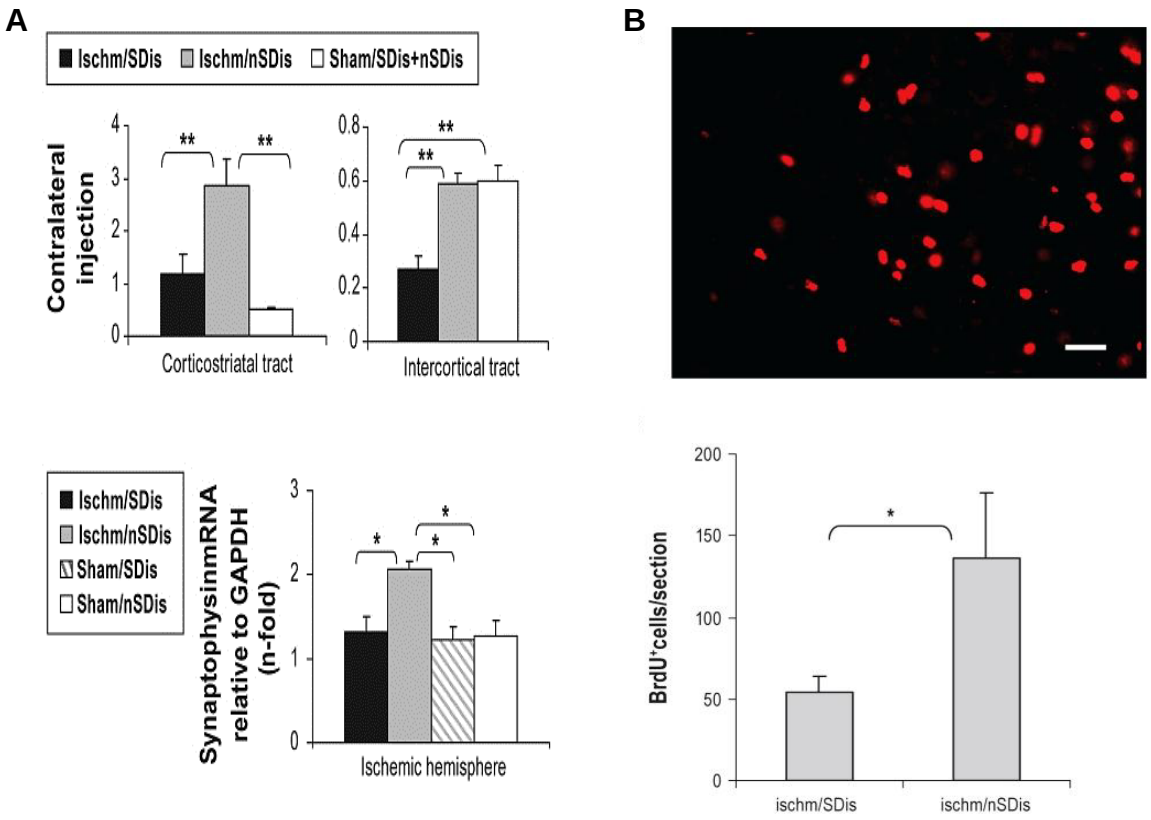

Figure 3.3.2 SDis-induced reduction in axonal sprouting, synaptogenesis and cell proliferation.

A. Effects of SDis on axonal sprouting of corticostriatal and corticocortical projection neurons in the contralateral hemisphere. Upper panel: quantification of BDA labeled corticostriatal (left panel) and intercortical (right panel) axons. Lower panel: expression of synaptophysin mRNA. *P<0.05, **P<0.01. **B.** Numerous newborn cells labeled with BrdU in the peri-infarct area, visualized by single immunofluorescence staining. Scale bar, 50 μm. n = 4 per group. **P<0.01 (redrawn from (Zunzunegui et al., 2011)).

Taken together, all of the mentioned experiments provide strong evidence that sleep might play a dual role in brain repair, promoting neuroprotection in the acute phase and/or enhancing neuroplasticity in the delayed phase after stroke. Therefore, it has been and still is of high interest to perform further experiments exploring the potential of sleep promotion for improving stroke outcome.

3.4 Sleep-promoting agents: baclofen and gamma-hydroxybutyrate

One of the most accessible methods to influence sleep is to use pharmacological approach. The administration of specific drugs inducing changes in sleep architecture and/or sleep intensity offer a promising tool to manipulate stroke outcome. Since gamma-aminobutyric acid (GABA) have been shown to play a major role in sleep regulation (Gottesmann, 2002), substances affecting GABAergic transmission may thus be used as an effective way to manipulate sleep.

Metabotropic receptors are the site of action for the majority of therapeutic agents used currently in clinical practice. Nevertheless, there are many receptors in this class that have not been yet therapeutically exploited, including $GABA_B$ receptors. The natural ligand for $GABA_B$ receptor is GABA, which is well established as the principal inhibitory neurotransmitter. The $GABA_B$ receptors are located in the brain both pre- and post-synaptically where they are coupled to Ca^{2+} and K^+ channels. Operating through second messengers, they cause increased outward K^+ or decreased Ca^{2+} conductance (Bowery, 2006). In consequence, their activation leads to various effects resultant from inhibition of the transmitter release and/or neuronal hyperpolarization. Hence, stimulation of these receptors can produce numerous outcomes that might be amenable to drug development. Additionally, GABA has been shown to be critical for cortical plasticity and sensory mapping. Altering GABAergic transmission modifies sensory maps during development (Hensch, 2005), and produces rapid alterations in adult cortical maps that resemble changes occurring after stroke (Foeller et al., 2005). Thus, GABAergic mechanisms may mediate changes in neuronal excitability that have a central role in functional recovery of the peri-infarct cortex after stroke.

Baclofen (Bac) is a structural analog of GABA mediating its effects directly via activation of the $GABA_B$ receptors. Historically, Bac was originally developed in the 1920s to treat epilepsy as a derivative of diazepam. Although its clinical effect on epilepsy appeared to be disappointing, it was found to be beneficial on diminishing the number and severity of muscle spasms. It has been therefore used in the United States since 1977 for treatment of spasticity in several disorders such as multiple sclerosis or cerebral palsy (Albright et al., 1991, Paisley et al., 2002, Bensmail et al., 2006).

Gamma-hydroxybutyric acid (GHB) is a naturally occurring compound in the mammalian brain that is both, a precursor and a degradation product of GABA. It is

found in micromolar concentrations (1–4 mM) in all regions of the mammalian brain and considered to act as a neuromodulator or neurotransmitter (Cash, 1994, Maitre, 1997). GHB binds with high affinity to its own GHB receptor, which was found to have the highest density in the hippocampus, cortex and the thalamus (Hechler et al., 1992, Castelli et al., 2000). However, accumulating evidence suggests that most of the pharmacological and clinical effects of exogenously administered GHB, including sedation, anaesthesia and sleep are mediated by activation of $GABA_B$ receptors (Carai et al., 2001, Kaupmann et al., 2003, Carai et al., 2008, Vienne et al., 2010). Therefore, it has been widely used in the clinical practice as an anaesthetic adjuvant (Kleinschmidt et al., 1999) and as a treatment for various sleep disorders, particularly narcolepsy with cataplexy (Scrima et al., 1989, Lammers et al., 1993, Fuller and Hornfeldt, 2003, Poryazova et al., 2011). Although GHB has been proposed as a biologically active neuromodulator (Maitre, 1997), the precise function of endogenous GHB in the brain is unknown.

Given that both, Bac and GHB act as $GABA_B$ receptor agonists, they share some commonality of action. For example, they have both found clinical use in the treatment of alcohol and opiate withdrawal and in promoting long-term abstinence from these drugs' action (Cousins et al., 2002). Importantly, both drugs differ in the binding affinity, i.e. GHB is a low-, while Bac is a high-affinity agonist of $GABA_B$ receptors (Lingenhoehl et al., 1999). Hence, there are some notable differences in the effects mediated by both drugs. Unlike GHB, Bac has been shown to have few euphoric effects, reduced abuse potential and no physical dependence (Kaupmann et al., 2003, Carter et al., 2009). Additionally, Bac is not used in anaesthesia and has been never reported to be effective in treatment of narcolepsy (Kaupmann et al., 2003). However, the functional correlates and cellular mechanisms contributing to these differential responses remain elusive.

The effects of Bac and GHB on stroke outcome in animals are controversial. Moreover, the effects of both substances have been evaluated only when the drug was administered during or before the acute phase of ischemic injury. It had been shown that Bac injections before or immediately after stroke had no beneficial effect on mortality, cellular damage and memory (Rosenbaum et al., 1990), whereas it was neuroprotective at the high dose when administered 30 min before (Zhang et al., 2007) or 5 min after (Jackson-Friedman et al., 1997) ischemia. Several laboratories have shown the neuroprotective effect of GHB (Vergoni et al., 2000, Ottani et al., 2003, Ottani et al.,

2004) after stroke. As mentioned already in section 3.3, recent study in mice showed that GHB administrated shortly after ischemia, accelerated functional recovery without significantly changing stroke morphology (Gao et al., 2008). However, the mechanisms underlying this GHB-induced beneficial effects on stroke outcome remain unclear.

Both, Bac and GHB have been shown to affect sleep. Administration of Bac and GHB has been demonstrated to increase NREM sleep duration and intensity in humans (Lapierre et al., 1990, Series et al., 1992, Van Cauter et al., 1997, Darbari et al., 2005, Huang and Guilleminault, 2009, Vienne et al., 2012) and to induce slow- wave sleep (SWS) in rats (Godschalk et al., 1977, Monti et al., 1979, Manfridi et al., 2001, Ulloor et al., 2004, Darbari et al., 2005, Datta, 2007, Huang and Guilleminault, 2009). Interestingly, both drugs have been also reported by recent studies to induce sub-anaesthetic state different from physiological sleep (Meerlo et al., 2004, Vienne et al., 2010). Taken together, some findings suggested physiological sleep-promoting effects and involvement in homeostatic sleep regulation of both drugs, while others contradicted them. Therefore, the effects of Bac and GHB on sleep and thus possible endorsement of recovery after ischemic insult remain still unclear.

3.5 Thesis aims

The overall goal of the current thesis was to investigate how alterations in sleep after ischemic stroke affect recovery processes in rats. Based on the previous studies providing a major insights about a possible beneficial role of sleep in brain repair (Gao et al., 2008, Gao et al., 2010, Zunzunegui et al., 2011, Cam et al., 2013), we hypothesized that promotion of sleep, and in particular of slow wave activity, could enhance endogenous mechanisms of neuronal plasticity, which would improve functional recovery. Sleep could serve not only as a clinically relevant new approach to facilitate neurological recovery after brain damages but also as a useful tool to examine plasticity under pathophysiological conditions such as stroke. Therefore, it is of high interest to further explore this unique, poorly understood research area and unveil the importance of sleep in brain restoration.

In order to examine our hypothesis, specific aims were addressed in two main experiments.

1. The aim of the first project **(Manuscript 1)** was to characterise the changes induced by Bac and GHB in sleep, EEG activity and behaviour in healthy rats. The main focus of this work was to explore whether each of the drug had sleep-promoting properties and therefore, possible therapeutic potential. Although several studies have investigated the effects of Bac and GHB on sleep in healthy humans and animals, their effects remain still unclear. In order to elucidate if the time of the day affected the action of the drugs, Bac and GHB were given at two different time points (beginning of the light and of the dark phase), corresponding to physiological circadian periods of rest and activity in rats.

2. The aim of the second project **(Manuscript 2)** was to evaluate the effects of sleep promotion with Bac on motor function recovery and endogenous mechanisms involved in neuronal plasticity (axonal sprouting and neurogenesis) after ischemic stroke. The choice of Bac was based on the data obtained in the first study. In order to investigate the drug effects on brain repairing processes, the targets of neurorestorative therapies, Bac was administered twice a day starting 24 h after ischemic injury for a period of 10 days. This delayed approach is of clinical significance since it falls out of the time

window available for acute neuroprotective treatments, which is within 6 h after ischemia onset. Furthermore, the plastic events that characterise the semi-acute phase after stroke diminish and slow with time. Therefore, an important challenge is to find ways to widen the therapeutic window and to keep it open for a longer period of time in order to optimise post-stroke recovery.

4. EXPERIMENTAL RESEARCH PART

4.1 Manuscript 1

Effects of baclofen and gamma-hydroxybutyrate on behaviour, EEG activity and sleep in rats.

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Contribution of **A. Hodor**:

study design, data collection, data analysis (except the EEG spectra analysis), interpretation of results, preparation of manuscript

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ABSTRACT

Study objectives: Animal and human studies have shown that sleep may have an impact on functional recovery after brain damage. Baclofen (Bac) and gamma-hydroxybutyrate (GHB) were shown to induce physiological sleep in humans, however, their effects in rodents are unclear. The aim of this study is to characterise sleep and EEG after Bac and GHB administration in rats.

Design: Rats were implanted with EEG/EMG electrodes for sleep recordings. GHB, Bac or saline were injected 1h after light and dark onset. Vigilance states and EEG spectra were quantified.

Settings: Basic sleep research laboratory.

Measurements and Results: Bac and GHB induced a non-physiological state characterised by atypical behaviour and abnormal EEG pattern. After termination of this state Bac was found to increase the duration of NREM and REM sleep, reduce sleep fragmentation and affect NREM sleep episode frequency and duration ($p<0.05$). GHB had no major effect on vigilance states. Bac increased EEG power density in NREM sleep in the frequencies 1.5-6.5 and 9.5-21.5 Hz compared to saline ($p<0.05$), while GHB enhanced power in 1-5 Hz and reduced in 7-9 Hz frequency band. SWA in NREM sleep was enhanced during first 1-2 h following termination of the non-physiological state. The magnitude of the drug effects was stronger during the dark phase.

Conclusion: In contrast to GHB, Bac facilitated and consolidated sleep and promoted EEG delta oscillations after the end of the drug effect. The effects of sleep promotion on functional recovery after stroke could be tested with baclofen in both humans and rats.

INTRODUCTION

Promoting sleep has been proposed as a promising new approach to facilitate functional recovery after brain damage (Carmichael and Chesselet, 2002, Gao et al., 2008, Bassetti and Hermann, 2011, Cam et al., 2013). Baclofen (Bac) and gamma-hydroxybutyrate (GHB) have been suggested to induce physiological sleep in humans, however, their effects in rodents are unclear.

Gamma-hydroxybutyric acid, an endogenous metabolite of gamma-aminobutyric acid (GABA), is found in all regions of the mammalian brain and considered to act as a neuromodulator or neurotransmitter (Cash, 1994, Maitre, 1997). High doses of exogenous GHB induce sedation, anaesthesia and sleep (Cash, 1994, Carai et al., 2001). Therefore, it has been widely used in the clinical practice as an anaesthetic adjuvant (Kleinschmidt et al., 1999) and as treatment for narcolepsy with cataplexy (Scrima et al., 1989, Lammers et al., 1993, Fuller and Hornfeldt, 2003, Poryazova et al., 2011). Although most of the pharmacological and clinical effects of exogenously administered GHB are mediated by $GABA_B$ receptors (Carai et al., 2001, Kaupmann et al., 2003, Carai et al., 2008, Vienne et al., 2010), GHB has its own endogenous receptors (Hechler et al., 1992, Castelli et al., 2000) with distinct distribution in the brain (Maitre, 1997, van Nieuwenhuijzen et al., 2009). Yet, the precise function of endogenous GHB remains unknown.

Baclofen is another $GABA_B$ receptor agonist, usually used to treat spasticity (Albright et al., 1991, Paisley et al., 2002, Bensmail et al., 2006). Although both drugs bind to $GABA_B$ receptors, GHB has a lower affinity than Bac (Lingenhoehl et al., 1999, Wu et al., 2004). It has been shown that GHB and Bac induce c-Fos expression in distinct brain regions (van Nieuwenhuijzen et al., 2009). Interestingly, only GHB administration activated brain areas involved in the regulation of sleep and reward processes. Behavioural effects of GHB and Bac are different. In contrast to GHB, Bac has reduced abuse potential, euphoric effects and does not lead to physical dependence (Kaupmann et al., 2003, Carter et al., 2009). Thus, it is not used in anaesthesia or in treatment of narcolepsy (Kaupmann et al., 2003).

Several studies have explored the effects of GHB and Bac on sleep in healthy humans and animals. It has been shown that GHB decreases sleep latency and increases deep slow-wave sleep (SWS) in humans (Lapierre et al., 1990, Series et al., 1992, Van Cauter et al., 1997, Vienne et al., 2012) and enhances SWS in rats (Godschalk et al., 1977,

Monti et al., 1979). Interestingly, GHB has been also reported to induce hypersynchronous electroencephalogram (EEG) activity in awake humans (Mamelak et al., 1977, Van Cauter et al., 1997) and animals (Godschalk et al., 1977, Stock et al., 1978, Meerlo et al., 2004). Bac increased non-rapid eye movement (NREM) sleep and promoted EEG delta waves during NREM sleep in humans (Darbari et al., 2005, Huang and Guilleminault, 2009, Vienne et al., 2012) and SWS in rats (Manfridi et al., 2001, Ulloor et al., 2004, Darbari et al., 2005, Datta, 2007, Huang and Guilleminault, 2009). The effects of Bac on rapid eye movement (REM) sleep remain controversial. Infusion of Bac into the pedunculopontine tegmental nucleus suppressed REM sleep in rats (Ulloor et al., 2004, Datta, 2007), whereas unilateral Bac infusion into the nucleus basalis of Meynert had no effect on REM sleep (Manfridi et al., 2001). Recent studies in mice have demonstrated that both drugs induce sub-anaesthetic state different from physiological sleep (Meerlo et al., 2004, Vienne et al., 2010). Altogether, it is still unclear whether Bac and GHB induce physiological sleep in rodents.

The aim of the present study was to investigate the effect of Bac and GHB on behaviour, vigilance states and EEG pattern in rats, to evaluate their sleep-promoting properties and possible therapeutic potential. To address a circadian influence on the magnitude of the effects, the drugs were injected at the onset of the light and dark phase, which corresponds to periods of rest and activity in the rats.

METHODS

Animals

Adult male Sprague-Dawley rats (n = 23; Harlan Laboratories, Netherlands), weighing 250-270 g at the beginning of the experiment, were maintained on a 12 h light-dark cycle (light onset at 08:00 or 09:00) and 22 ± 0.5°C ambient temperature. They were kept individually in Macrolon cages and provided with food and water *ad libitum.* The experiments were carried out with governmental approval according to local guidelines for the care and use of laboratory animals in the University Hospital Zürich, Switzerland (where A.H., S.P., B.G. and C.B. worked, when the experiments were conducted).

Surgery

All rats were implanted epidurally with EEG and electromyogram (EMG) electrodes under deep anaesthesia (2% isoflurane in 30% O_2 and 70% N_2O). Four gold-plated mini-screws were positioned in the skull over the motor cortex of the right and left hemispheres (+/-2 mm to bregma, 2 mm lateral to midline). Electrodes were connected to stainless steel wires and fixed to the skull with dental cement. Two gold wires were inserted bilaterally in the neck muscles for EMG recording. At least 8-10 days were allotted for recovery from surgery before the experiment.

Drugs

GHB (Xyrem® - Sodium Oxybate, donated by UCB-Pharma) and Bac (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) were diluted in saline (0.9% NaCl) to obtain 100 mg/ml GHB and 3 mg/ml Bac stock solutions. Bac (10 mg/kg) and two doses of GHB (150 or 300 mg/kg) were administered intraperitoneally (i.p.). The doses were chosen based on the previous studies (Meerlo et al., 2004, Koek et al., 2005, Vienne et al., 2010).

Experimental protocol

Rats were subdivided into four treatment groups, including Bac-10 (n = 6), GHB-150 (n = 6), GHB-300 (n = 5), and saline (n = 6; Fig. 4.1.1). Drugs were injected 1 h after light and dark onset. Each rat received two injections (one during the light phase and one during the dark phase) 2-3 days apart to allow drug washout. After each injection rats were visually observed by the investigator and their behaviour was video recorded. 24-h EEG and EMG recordings were performed during baseline and after drug administration.

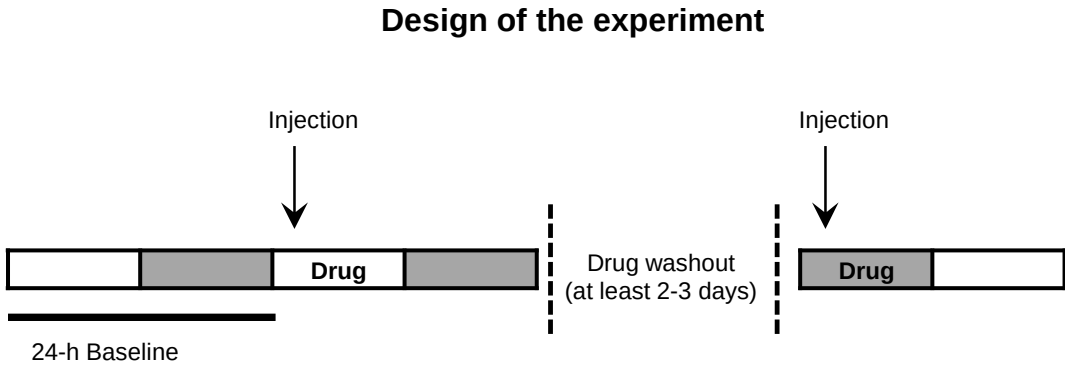

Figure 4.1.1
EEG and EMG were recorded during a 24-h baseline day and following drug injections performed 1 h after light and dark onset. Every rat received two intraperitoneal injections. At least 2-3 days were allowed for drug washout between injections. Four treatment groups were designed: Baclofen 10 mg/kg (Bac-10, n = 6), GHB 150 mg/kg (GHB-150, n = 6), GHB 300 mg/kg (GHB-300, n = 5) and saline (n = 6). 12-h light and 12-h dark phase are indicated by white and grey bars, respectively.

EEG recording and analysis

EEG and EMG were sampled at 200 Hz, signals were amplified, filtered and converted into analog-to-digital signals. Hardware EMBLA and Somnologica-3 software (Medcare Flaga, Iceland) were used. Activity in the 50 Hz band was discarded from the analysis because of power line artifacts. Power spectra of the EEG were obtained by a discrete Fourier transformation (range: 0.75–25 Hz; frequency resolution: 0.25-Hz bins; time resolution: consecutive 4 sec epochs; window function: hamming). Three vigilance states - NREM sleep, REM sleep and wakefulness - were scored visually with 4-sec epochs. Standard criteria were used to identify vigilance states (Tobler et al., 1997). Epochs were assigned to a specific vigilance state when more than half of the epoch fulfilled the criteria for that state. Epochs containing EEG artifacts were excluded from spectral analysis in both derivations (14% of recording time, most of them (11%) occurred during wakefulness). In addition to conventional vigilance states we introduced two new states resembling sleep and wakefulness following administration of the drugs ("drug-induced states"). These states were characterised by atypical behaviour and abnormal hypersynchronous EEG pattern. The first 4 sec epoch following drug administration was taken as the onset of the non-physiological vigilance state and the last epoch of abnormal EEG (determined by visual inspection of EEG) was taken as the end of the state. Our analysis was focused on the time period following the end of this state.

Three recording periods were scored and evaluated for every animal: 24-h baseline, 11 h following injection during the light phase and 11 h following injection during the dark phase.

Statistical analysis

Drug and time of day effects were evaluated by a repeated-measures or mixed-models analysis of variance ANOVA (SAS software, SAS Institute, Cary, NC, USA). Post-hoc paired and unpaired *t*-test, Tukey-Kramer, Bonferroni and Kruskal-Wallis tests were performed if the results of the ANOVA reached statistical significance ($p<0.05$). All reported values are means ± SEM.

RESULTS

Effects of baclofen on behaviour, vigilance and EEG

Behaviour and vigilance states. We first studied the effect of Bac administration on sleep-wake behaviour. After injection of a single 10 mg/kg dose of Bac (Bac-10), rats were lying down immobile, their body posture was unnaturally flat with limbs stretched sideways, while the eyes remained open (Fig. 4.1.2A). This behavioural response occurred 5-10 min after drug injection and lasted for 40-70 min. Circadian time of the drug administration had no effect on behavioural response. Concomitant to this behavioural response, we found that Bac-10 induced specific EEG pattern characterised by hypersynchronous slow waves (Fig. 4.1.2B), which progressively predominated EEG. The "drug-induced" state lasted 246 ± 38 and 216 ± 24 min during the light and dark phase, respectively.

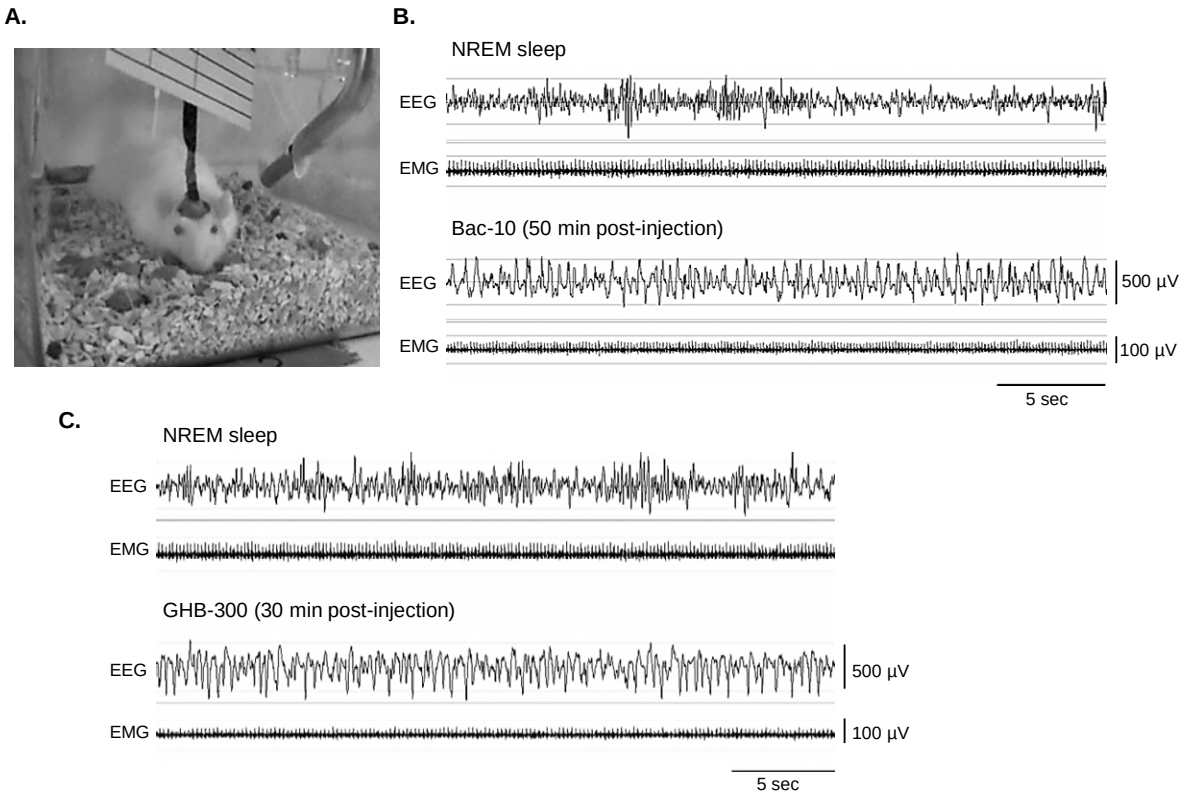

Figure 4.1.2 Effect of baclofen 10 mg/kg (Bac-10) and GHB 300 mg/kg (GHB-300) on behaviour and EEG pattern.
A. The picture of the rat 30 min after Bac-10 administration: atypical flat body posture with eyes open. **B.** Representative 30 sec raw EEG and EMG traces during physiological NREM sleep and following Bac-10 administration in rats. **C.** Representative 30 sec raw EEG and EMG traces during physiological NREM sleep and following GHB-300 administration in rats.

When acute effects of the drug on behaviour and EEG vanished, normal vigilance states could again be determined. Therefore, long-term effects of the drug on the total amount of wakefulness, NREM sleep, REM sleep and corresponding EEG spectra were evaluated after the termination of the non-physiological ”drug-induced” state. We found that NREM sleep was increased during the light and dark phase compared to the corresponding baseline values ($p<0.05$, paired *t*-test; Table) and during the dark phase compared to the saline treatment ($p<0.005$, unpaired *t*-test; Fig. 4.1.3A). The drug effect on NREM sleep was stronger during the dark phase (91.4 ± 8.1 vs. 15.7 ± 6.0 min, $p<0005$, paired *t*-test; values were computed as a difference between treatment and corresponding baseline). The duration and frequency of NREM sleep episodes were affected by the timing of Bac administration. Thus, during the light phase Bac-10 increased the duration of the episodes ($p<0.01$, paired *t*-test; Fig. 4.1.3B), but reduced their frequency ($p<0.05$), while during the dark phase it increased the frequency of the

episodes ($p<0.0001$) without changing their duration. Moreover, the number of NREM sleep and waking episodes (not shown) was significantly different from saline treatment in the dark phase ($p<0.01$, unpaired *t*-test; Fig. 4.1.3C). In addition, Bac-10 administered during the dark phase reduced sleep fragmentation (defined as the occurrence of waking episodes < 16 sec per hour of sleep) compared to corresponding baseline value (43.1 ± 3.9 vs. 83.9 ± 4.3, respectively; $p<0.001$, paired *t*-test) or to the saline treatment (40.9 ± 5.3 vs. 16.9 ± 12.1, respectively; $p<0.01$, unpaired *t*-test; values represent difference between treatment and corresponding baseline). Finally, we further found that REM sleep was enhanced during the dark phase only, compared to the corresponding baseline and saline values ($p<0.05$; Table 4.1.1 and Fig. 4.1.3A).

Drug		Wakefulness		NREM sleep		REM sleep	
		BL	Treatment	BL	Treatment	BL	Treatment
Bac-10	L	125.14±10.82	110.53±15.27	230.44±23.37	246.1±19.64*	58.34±5.95	57.3±4.45
	D	323.18±7.22	220.08±13.76**	100.86±14.57	192.29±19.19**	19.56±4.41	31.22±3.82*
GHB-150	L	195.83±12.53	173.79±10.18	337.59±12.49	357.34±10.09	77.11±8.69	79.40±7.81
	D	378.11±20.21	361.49±23.02	196.76±17.81	207.34±18.25	34.03±6.33	40.07±5.73
GHB-300	L	195.97±13.42	180.72±11.20	307.40±7.22	313.41±4.33	59.33±5.73	68.51±6.18
	D	359.27±21.03	330.79±20.02	205.97±12.22	233.00±10.35	48.57±9.29	49.99±4.54
Saline	L	236.19±12.33	229.66±13.89	347.90±10.38	346.20±13.25	75.91±4.98	84.14±5.04*
	D	432.84±24.50	422.04±15.93	195.27±21.39	206.54±13.81	31.89±3.93	31.41±3.69

Table 4.1.1 Effects of Bac and GHB on vigilance states
Amount of wakefulness, NREM sleep and REM sleep (in minutes) following Baclofen (Bac), GHB and saline administration during the light (L) and dark (D) phase. The amount of vigilance states was computed for the period after the end of the non-physiological vigilance states and compared with the corresponding time-matched baseline (BL) values. Rats were assigned to four treatment groups: Bac 10 mg/kg (Bac-10, n = 6), GHB 150 mg/kg (GHB-150, n = 6), GHB 300 mg/kg (GHB-300, n = 5) and saline (n = 6). Asterisks indicate differences between BL and treatment: *$p<0.05$, **$p<0.001$, paired *t*-test.

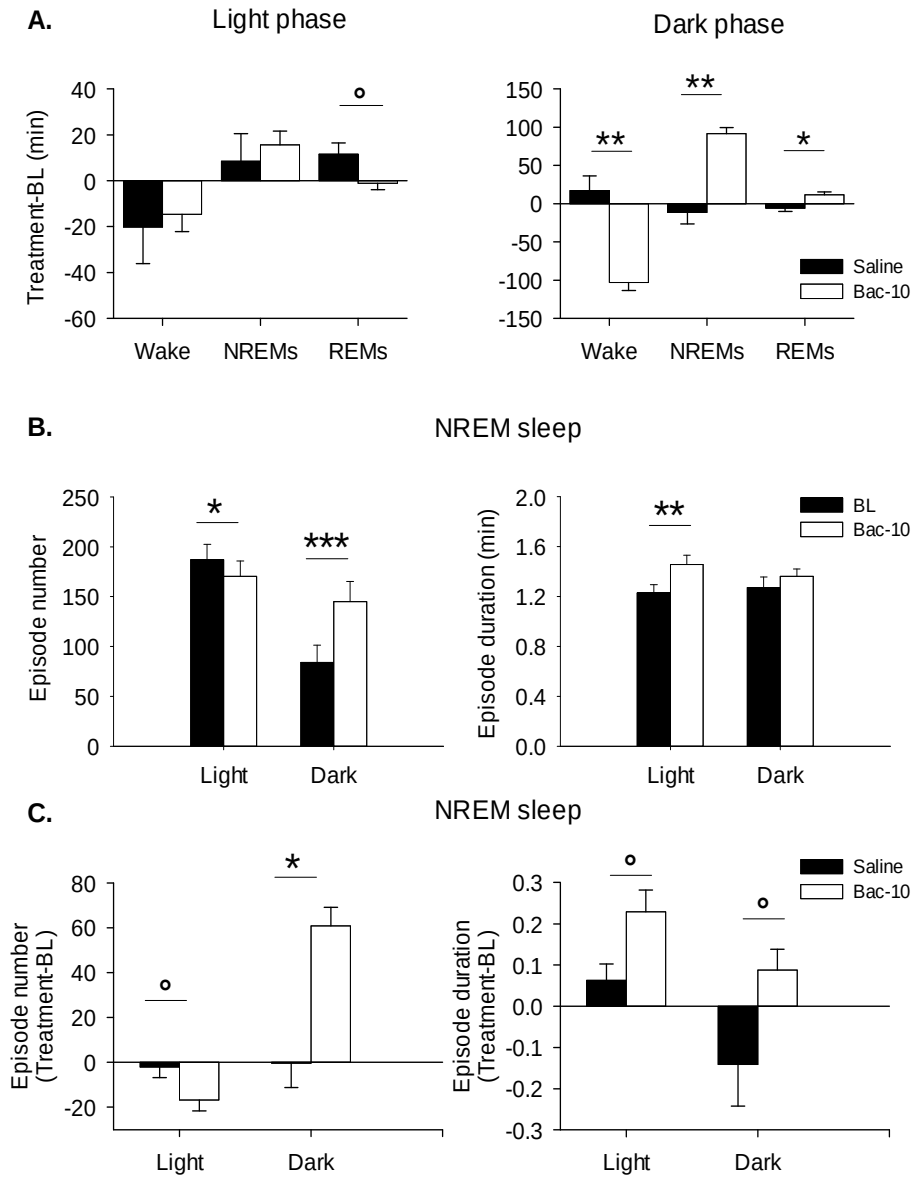

Figure 4.1.3 Effect of baclofen 10 mg/kg (Bac-10) on vigilance states.

A. Amount of wakefulness (Wake), NREM sleep (NREMs) and REM sleep (REMs; in minutes) following Bac-10 (n = 6) administration during the light and dark phase compared to the saline treatment (n = 6). Mean ± SEM values were computed as a difference between treatment and corresponding baseline (BL) for the period after the end of the non-physiological vigilance states. Bac-10 vs. saline: °p<0.1, *p<0.05, **p<0.005, unpaired *t*-test. **B.** Number and duration (in minutes) of NREM sleep episodes following Bac-10 administration during the light and dark phase (baseline comparison). Mean ± SEM values were computed as a difference between treatment and corresponding time-matched BL for the period after the end of the non-physiological vigilance states. Treatment vs. BL: *p<0.05, **p<0.01, ***p<0.0001, paired *t*-test. **C.** Number and duration (in minutes) of NREM sleep episodes following Bac-10 and saline administration during the light and dark phase (saline comparison). Values were computed as a difference between treatment and corresponding time-matched BL for the period after the end of the non-physiological vigilance states. Treatment vs. saline: °p<0.1, *p<0.01, unpaired *t*-test.

EEG spectra and slow-wave activity (SWA) in NREM sleep. Interestingly, we found that Bac-10 administration induced prominent changes in the EEG spectra during NREM sleep. EEG power density in NREM sleep was increased significantly above corresponding time-matched saline values in the 2.25-5.5 and 10.75-14.75 Hz frequency bands during both the light and dark phase ($p<0.05$, unpaired *t*-test; Fig. 4.1.4A). Moreover, power increase was significantly higher after drug injection in the dark phase compared to the one in the light phase in the frequencies above 3.25 Hz ($p<0.05$, paired *t*-test; data were normalised to the 12-h baseline light phase; not shown). In waking, significant reduction of EEG power was observed after Bac-10 administration compared to saline in the dark phase (1-20.5 Hz; Fig. 4.1.4B). No such effect was observed in the light phase. Again, Bac administration induced stronger power reduction during the dark phase compared to the light phase in the frequencies between 1.25-6.75 and 8.25-18.25 Hz ($p<0.05$, paired *t*-test; not shown). Bac-10 also affected EEG activity during REM sleep (Fig. 4.1.4C). It enhanced power density in theta (6.5-8.75 Hz) and beta (above 20.75 Hz) frequencies in the dark phase compared to saline. The light-dark power difference was found in the frequencies 6.75-7.5 Hz ($p<0.05$, paired *t*-test; not shown).

Further we show that Bac-10 increased sleep intensity based on the analysis of the time course of SWA (0.75-4 Hz) in NREM sleep. SWA was enhanced above the saline level during first two hours after light or dark Bac-10 injection (Fig. 4.1.4D). The Bac-10 effect was stronger during the dark phase ('phase' $p<0.05$, Tukey-Kramer after mixed ANOVA factor 'phase' $F_{(1,15)}=5.36$, $p=0.0351$; not shown).

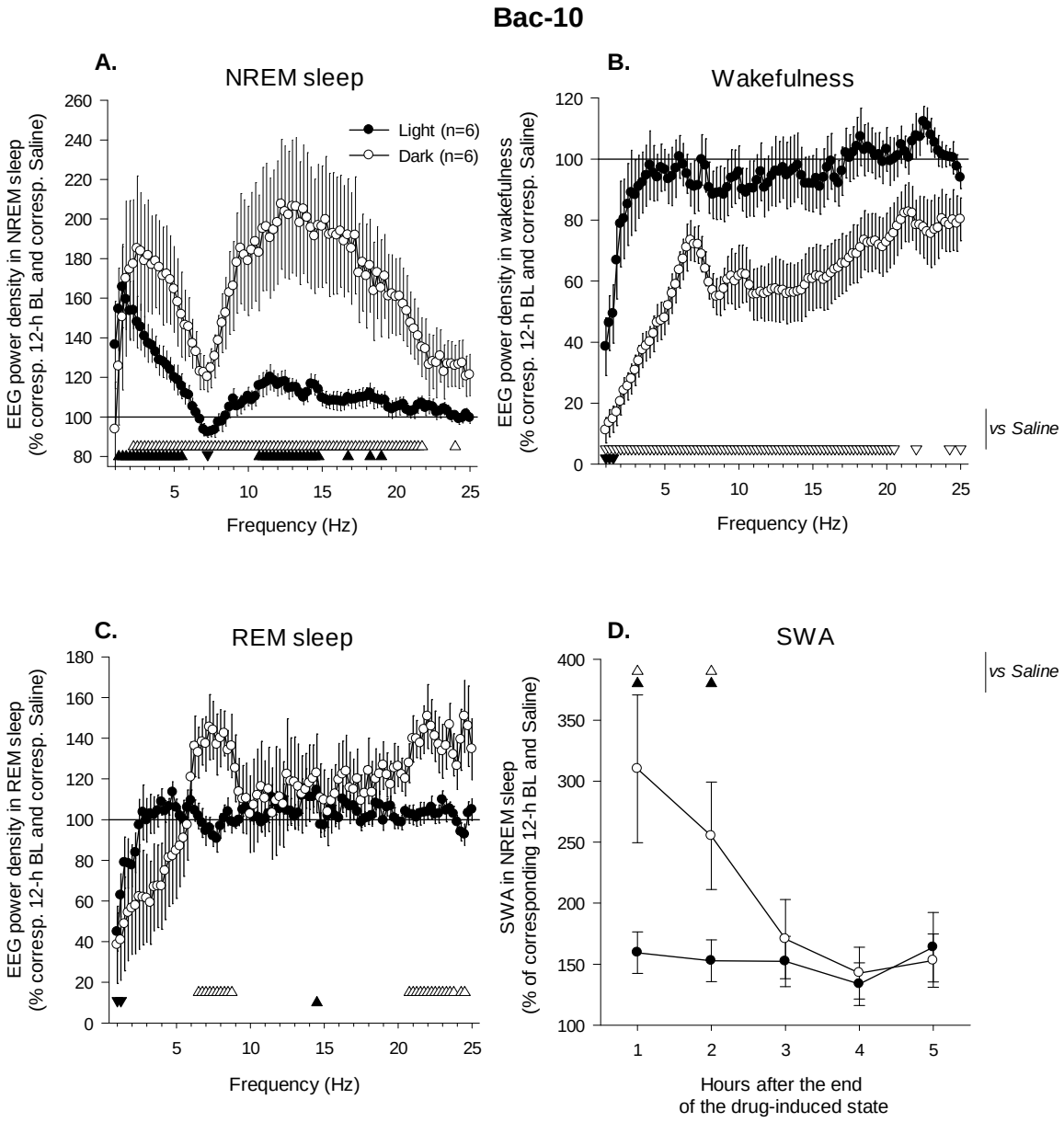

Figure 4.1.4 Effect of baclofen 10 mg/kg (Bac-10) on EEG spectra.
EEG power density in NREM sleep **(A)**, wakefulness **(B)** and REM sleep **(C)** following Bac-10 (n = 6) administration during the light (filled circles) and dark phase (open circles). Power in each frequency bin after Bac-10 or saline treatment (n = 6) was first normalised to the corresponding mean 12-h light or dark baseline value of the same bin. Thereafter Bac-10 values were expressed as percentage of power after saline treatment. The curves connect mean values ± SEM computed for the period after the end of the non-physiological vigilance states. **D.** Time course of slow-wave activity (SWA) in NREM sleep following Bac-10 administration during the light (filled circles) and dark phase (open circles). Mean ± SEM 1-h values were first expressed as percentage of the corresponding 12-h light or dark baseline SWA in NREM sleep and then as percentage of SWA in NREM sleep after saline treatment. Differences between Bac-10 and saline during the light and dark phase are indicated by black and white triangles, respectively; orientation of the triangles points to the direction of the difference: $p<0.05$ (unpaired *t*-test following significant ANOVA).

Effects of GHB on behaviour, vigilance and EEG

Behaviour and vigilance states. We then studied the effect of GHB administration on sleep-wake behaviour using two different doses (150 mg/kg and 300 mg/kg). We found that administration of GHB-300 resulted in a similar behavioural response as Bac-10. GHB-150 did not affect animal behaviour (rats remained awake, responded to stimuli and moved around the cage). Similar to Bac-10, GHB-300 induced abnormal, hypersynchronous EEG pattern (Fig. 4.1.2C). GHB-150 administration was followed by bursts of hypersynchronous slow waves appearing irregularly. Therefore, our results suggest that GHB induced behavioural state distinct from physiological sleep or wakefulness. The duration of non-physiological states depended on the dose applied. The states lasted 50 ± 10 and 51 ± 5 min after GHB-150 (light and dark phase, respectively) and 97 ± 6 and 46 ± 19 min after GHB-300.

When acute effects of the drugs on behaviour and EEG vanished, normal vigilance states could again be determined. Thus, long-term effects of the drugs on vigilance states and EEG spectra were evaluated after the termination of the non-physiological ”drug-induced” state. We found that GHB had no major effect on the amount of NREM sleep, REM sleep or wakefulness (Table 4.1.1).

EEG spectra and SWA in NREM sleep. We found that GHB application increased EEG power density in NREM sleep in the frequencies encompassing SWA range. However, significance was reached only in the dark phase after GHB-150 (Fig. 4.1.5A) and in the light phase after GHB-300 administration (Fig. 4.1.5B). Both doses of GHB affected waking EEG power in the dark phase. GHB-150 inhibited power only in the low frequencies (Fig. 4.1.5A), while the power in the almost entire frequency range was reduced by GHB-300 (Fig. 4.1.5B). GHB had no effect on waking spectra during the light phase. REM sleep EEG spectrum was increased in 7.75-9.5 Hz after GHB-150 administered in the dark phase (Fig. 4.1.5A). Minor light-dark differences in the EEG power were observed in the frequencies encompassing 4-4.25 Hz in NREM sleep, 4.5-6.25 and 8.75-13.25 in REM sleep and 5.25-6.5 in waking ($p<0.05$, paired *t*-test) after GHB-300 injections (not shown).

Recovery from drug effects was also assessed at the level of delta power in NREM sleep. Significant increase of SWA in NREM sleep was observed after GHB-300. SWA was above saline values during first 1-h interval following drug administration in the light

phase ($p<0.05$; Fig. 4.1.5C). GHB-150 had no significant effect on sleep intensity (not shown).

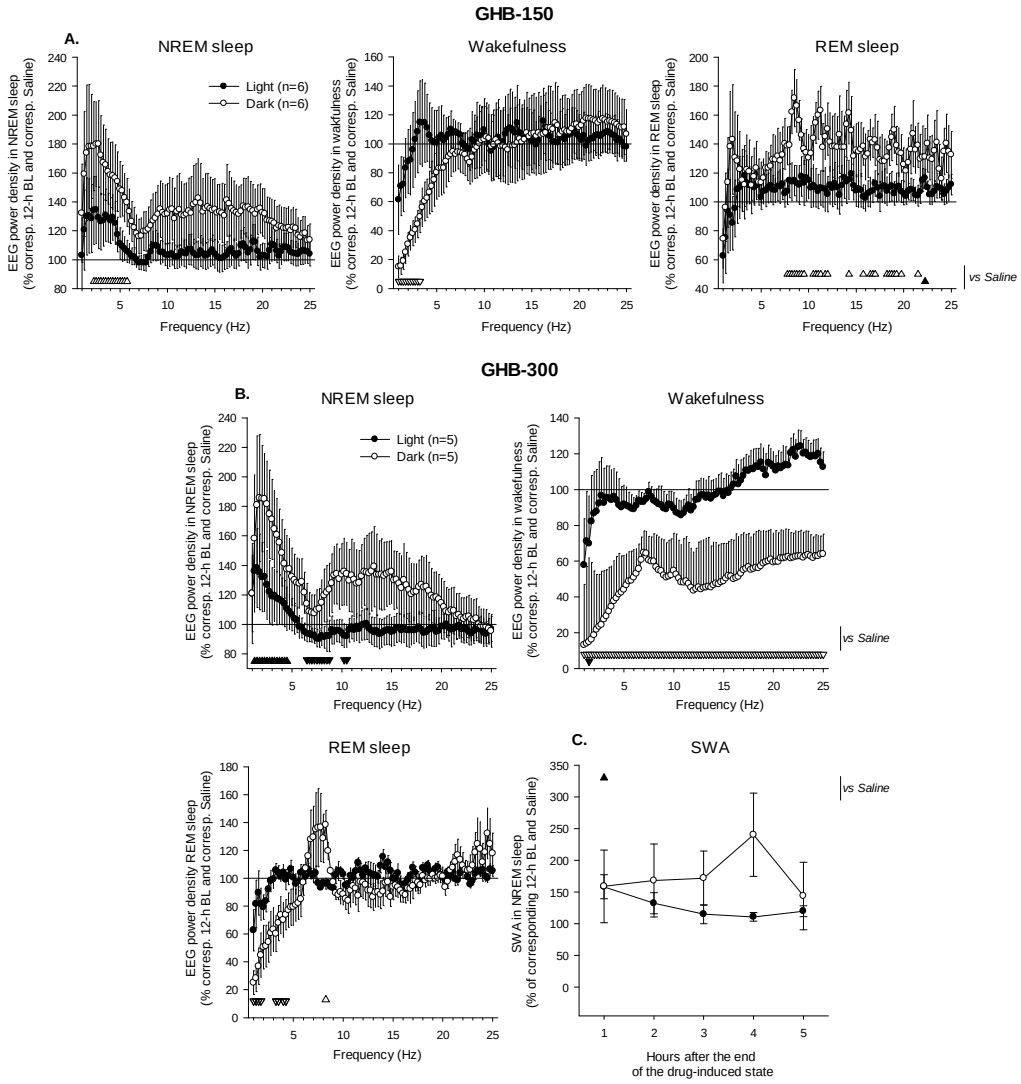

Figure 4.1.5 Effect of GHB 150 mg/kg (GHB-150) and 300 mg/kg (GHB-300) on EEG spectra.
A. EEG power density in NREM sleep, wakefulness and REM sleep following GHB-150 (n = 6) administration during the light (filled circles) and dark phase (open circles). **B.** EEG power density in NREM sleep, wakefulness and REM sleep following GHB-300 (n = 5) administration during the light (filled circles) and dark phase (open circles). Power in each frequency bin after GHB-150, GHB-300 or saline treatment (n = 6) was first normalised to the corresponding mean 12-h light or dark baseline value of the same bin. Thereafter GHB-150 or GHB-300 values were expressed as percentage of power after saline treatment. The curves connect mean values ± SEM computed for the period after the end of the non-physiological vigilance states. Differences between GHB-150 or GHB-300 and saline treatment during the light and dark phase are indicated by black and white triangles, respectively; orientation of the triangles points to the direction of the difference: $p<0.05$ (unpaired *t*-test following significant ANOVA). **C.** Time course of slow-wave activity (SWA) in NREM sleep following GHB-300 administration during the light (filled circles) and dark phase (open circles). Mean ± SEM 1-h values were first expressed as percentage of the corresponding 12-h light or dark baseline SWA in NREM sleep and then as percentage of SWA in NREM sleep after saline treatment. Difference between GHB-300 and saline during the light phase is indicated by black triangle ($p<0.05$, unpaired *t*-test following significant ANOVA).

DISCUSSION

Our data show that Bac and GHB robustly altered behaviour, vigilance states and EEG frequencies in rats. In particular, pharmacological manipulation resulted in a remarkable increase of EEG power in NREM sleep below 5 Hz after the termination of the drug state, pointing to the ability of the drugs to stimulate mechanisms generating EEG NREM sleep oscillations. EEG power in NREM sleep in the higher frequencies was also enhanced by both drugs. Moreover, we show for the first time that circadian time of Bac administration affected the response of the rats to the drugs. Specifically, treatment at the beginning of the dark phase induced larger changes in vigilance states and EEG spectra. Our results confirm and extend previous studies investigating the effects of Bac and GHB on changes in sleep and EEG pattern in mice (Meerlo et al., 2004, Vienne et al., 2010).

In animals, like in humans sleep is homeostatically regulated and the EEG SWA in NREM sleep is used as an indicator of sleep intensity (Borbely, 1982). Our analysis showed that Bac and both doses of GHB increased NREM sleep intensity after the end of the drug effect. Pharmacological manipulation affected not only EEG, but also led to the increase of sleep duration and consolidation. We cannot exclude that the increase of sleep and its intensity were a consequence of prolonged drug-induced state, during which sleep pressure continued to accumulate. The increase of SWA following Bac-10 was comparable to the increase observed after 6-h sleep deprivation (Vyazovskiy et al., 2007). However, drug-induced states were relatively short and lasted on average < 4 h. Moreover, duration of the drug state did not predict the SWA increase (Bac, n = 12: r=0.11, n.s.). Therefore, it is unlikely that "drug-induced" state was responsible for the increased sleep intensity observed after the state termination.

NREM sleep EEG changes were not restricted to the low frequencies. Thus, Bac-10 enhanced power density in the frequency range encompassing spindles (10.5-21.5 Hz), that might point to the increased EEG synchronization within the thalamo-cortical circuit (Terrier and Gottesmann, 1978). GHB-300 reduced power in the frequencies between 6.5-8.75 Hz.

It is well known that rodents, in contrast to humans, have a polyphasic sleep-wake pattern. Thus, rats exhibit more sleep during the light phase. Moreover, SWA in NREM sleep is usually high at the beginning of the light phase and reaches its nadir during the dark phase. In this study we applied Bac and GHB at two circadian time

points, which are characterised by high and low sleep pressure, respectively. Our data indicate that drugs administration during low sleep pressure conditions induced stronger changes in sleep and EEG power in NREM sleep. In contrast, treatment at the beginning of the light phase was less effective, probably due to a ceiling effect. Sleep consolidation (i.e. duration and frequency of NREM sleep episodes) was affected differently in the light and dark phase (Fig. 4.1.3B, C). The timing of drug administration influenced also EEG spectra. Light-dark differences were more prominent in waking EEG. Both drugs reduced EEG power in the dark phase (Figs. 4.1.4B and 4.1.5A, B).

Pharmacokinetics of the drugs might depend on the circadian phase and/or the level of animal activity. Indeed, there are data showing that kinetics can be modified according to the time of drug administration (Baraldo, 2008). Moreover, faster metabolism and, therefore, more transient effect of the drug are expected during the activity phase. That was not the case in our study. In addition, fluctuations in the level of neurotransmitters might play a role. Hence, it is important to consider intrinsic sleep need as well as drug kinetics during drug application in experimental and therapeutic conditions.

Consistent with previous studies in mice (Meerlo et al., 2004, Vienne et al., 2010), we showed that both GHB and Bac induced a drug state distinct from physiological sleep also in rats. Several criteria defining normal physiological sleep (Borbely and Tobler, 1989) were affected by the drugs. Specifically, rats consistently displayed unnatural body posture and revealed atypical hypersynchrony in the cortical EEG signal. The magnitude and duration of the observed effects depended on the drug and changed in a dose-dependent manner. The differences in the level of drug response between GHB and Bac are consistent with the half-life of the drugs in rats (60 min for GHB (Snead, 1977, Kueh et al., 2008) and 3-4 h for Bac (Lal et al., 2009)).

In our study GHB treatment had no effect on the amount of vigilance states. This is in line with results shown previously by Meerlo et al. (Meerlo et al., 2004). In contrast, recent study in mice reported decreased NREM and REM sleep in the light period following 300 mg/kg of gamma-butyrolactone, a prodrug of GHB (Vienne et al., 2010). Godschalk et al. (Godschalk et al., 1977) showed that doses of 50-100 mg/kg GHB increased SWS, while dose of 200 mg/kg induced EEG hypersynchrony and behavioural arrest. Studies investigating dose- and concentration-response relationship of GHB applied intravenously reported lack of the effect at 150 (Van Sassenbroeck et al., 2001)

or 200 mg/kg (Felmlee et al., 2010). Therefore, literature data describing the effects of GHB on sleep are contradicting. The discrepancy might be related to experimental conditions, including injection time, amount of data entering the analysis, route of drug administration, species.

Interestingly, atypical behavioural state and changes in EEG produced by Bac and GHB-300 have different time course. Thus, abnormal EEG pattern appeared few minutes after drug administration and lasted up to 97 min for GHB-300 or 5.5 h for Bac-10, greatly exceeding behavioural response (40-70 min for both drugs). Drug response might depend on absorption, distribution and elimination rates. After reaching peak plasma concentration the behavioural effects gradually dissipate, while longer-lasting effects in the brain can remain. Therefore, lower dose of GHB (150 mg/kg) was insufficient to cause any visible behavioural effects, but sufficient to induce changes in EEG.

Both drugs affected EEG power density in REM sleep. Significant increase of power in the theta frequency range was observed after GHB-150 (7.75-9.5 Hz) and Bac-10 (6.5-8.75 Hz) administration in the dark phase (Figs. 4.1.5A and 4.1.4C). There is evidence that median raphe nucleus (MRN) may serve to desynchronize hippocampal EEG and thus to block theta rhythm (Vertes, 1981). Bac, through $GABA_B$ receptors, might suppress firing of serotonergic neurons in the raphe nucleus (Colmers and Williams, 1988, Innis et al., 1988) and promote theta rhythms generation. Indeed, Bac infused into the serotonin-containing MRN promoted theta rhythm in anaesthetised rats (Varga et al., 2002, Li et al., 2005a). A number of studies reported increased serotonergic activity, including firing rate (Inouye and Kawamura, 1979), c-Fos immunoreactivity (Janusonis and Fite, 2001) and serotonin release (Kalen et al., 1989, Rueter and Jacobs, 1996) during dark phase. Interestingly, in our study enhanced theta power was present only during the dark phase.

In addition, GHB and Bac may affect other monoaminergic systems. Thus, both drugs inhibited noradrenergic neurons in locus coeruleus (Osmanovic and Shefner, 1988, Szabo et al., 2004) and dopaminergic neurons in ventral tegmental area (Madden and Johnson, 1998, Cruz et al., 2004) in rats. It has been suggested that a decrease of dopaminergic or noradrenergic transmission induces an increase of EEG spectral power (Sebban et al., 1999), while stimulation of noradrenergic neurons blocks slow cortical oscillations (Steriade et al., 1993). Moreover, a number of studies reported daily

rhythmicity in the activity of those systems with the peak in the middle of the dark phase (Nagayama, 1999, Feenstra et al., 2000), which may influence the action of both tested drugs.

In conclusion, our data show that 1) GHB and Bac induce a non-physiological resting state and affect vigilance, EEG and behaviour in rats, 2) these effects are dependent on the time of drug administration, 3) Bac, but not GHB, has sleep-promoting properties. It is possible that observed effects may be related to a complex mechanism, engaging multiple neurotransmitter systems. The effects of sleep promotion on functional recovery after stroke, what actually motivated this study, could now be tested with baclofen in rats.

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4.2 Manuscript 2

Baclofen facilitates sleep, neuroplasticity and recovery after stroke in rats.

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Contribution of **A. Hodor**:

study design, data collection, data analysis (except the EEG spectra analysis), interpretation of results, preparation of manuscript

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ABSTRACT

Sleep disruption in the acute phase after stroke has detrimental effects on recovery in both humans and animals. Conversely, the effect of sleep promotion remains unclear. The aim of this study was to investigate the effect of baclofen (Bac), considered a sleep-promoting drug, on stroke recovery in a rat model of focal cerebral ischemia (isch).

Rats, assigned to three experimental groups (Bac/isch, saline/isch or Bac/sham), were injected twice daily for 10 consecutive days with Bac or saline starting 24 h after induction of stroke. Sleep and functional recovery were assessed by EEG recordings and single pellet reaching test (SPR), respectively. To identify potential underlying mechanisms, axonal sprouting and neurogenesis were evaluated.

Repeated Bac treatment after ischemia increased NREM sleep amount during the dark phase. Moreover, ischemic rats treated with Bac performed significantly better in the SPR test than saline-treated animals. Axonal sprouting in the ipsilesional motor cortex and striatum, and neurogenesis in the peri-infarct region were significantly increased in the Bac/isch group.

In conclusion, delayed repeated Bac treatment after stroke increased NREM sleep and promoted both neuroplasticity and functional outcome. These data support the hypothesis of the role of sleep as a modulator of post-stroke recovery.

INTRODUCTION

Ischemic stroke is one of the most prevalent neurological conditions and a leading cause of death and long-term disability worldwide (Duncan, 2002, Burns et al., 2009). Despite progress made in understanding the mechanisms involved in neuronal damage during ischemia, limited advances have been reached in developing effective treatments for stroke patients (Ernst, 1990, Ottenbacher and Jannell, 1993). Spontaneous partial recovery after stroke is associated with neuronal plasticity mechanisms. Data from both patients and animal models showed a remodelling of neuronal networks in the hemisphere affected by stroke as well as recruitment of additional circuits from the contralesional hemisphere (Wiessner et al., 2003, Gerloff et al., 2006). Therefore, methods inducing or enhancing neuronal plasticity processes in the lesioned brain area may represent a novel effective therapeutic strategy for stroke.

There is growing evidence suggesting an important role of sleep in facilitating brain plasticity (Tononi and Cirelli, 2003, Vassalli and Dijk, 2009). Changes in sleep and sleep electroencephalogram (EEG) may reflect changes in connectivity within cortical neuronal network, but also drive changes in synaptic strength (Huber et al., 2004, Vyazovskiy et al., 2009). Sleep deprivation (SD) impairs induction and/or maintenance of long-term potentiation (LTP) (e.g. (McDermott et al., 2003)), a basic mechanism thought to underlie neuronal plasticity and memory formation, whereas sleep oscillations induced LTP (Chauvette et al., 2012). Moreover, it has been shown that plasticity-related genes and proteins display differential expression in sleep, wakefulness and following SD (Cirelli et al., 2004).

There is accumulating evidence suggesting an important role for sleep in stroke recovery. Manipulation of sleep after ischemia may in fact affect stroke outcome. Disruption of sleep during acute and subacute phase of stroke aggravated brain damage (Gao et al., 2010) and impeded functional recovery in rats (Zunzunegui et al., 2011). On the contrary, administration of gamma-hydroxybutyric acid (GHB), considered a sleep-promoting drug, immediately after reperfusion accelerated motor function recovery in mice (Gao et al., 2008). Furthermore, a physiological enhancement of sleep (following previous sleep deprivation) occurring immediately after stroke induction, was also associated with a reduction of brain damage (Cam et al., 2013). Sleep may, therefore, play a dual beneficial role in brain repair, fostering neuroprotection in the acute phase and enhancing neuroplasticity in the delayed phase after stroke.

The aim of the current study was to investigate the effect of sleep enhancement on brain repair and functional recovery after the acute phase of ischemic stroke. We hypothesized that induction of sleep or synchronized neuronal activity would facilitate motor function recovery and brain repair mechanisms in a rat model of focal cerebral ischemia. Considering our strong interest in translational approaches, we decided to use baclofen (Bac), a $GABA_B$ receptor agonist which is known to promote sleep in humans (Darbari et al., 2005). Our group has recently shown that Bac also increases non-rapid eye movement (NREM) sleep duration in rats (Hodor et al., in review).

METHODS

Animals

Adult male Sprague-Dawley rats (n = 53; Harlan Laboratories Netherlands, Charles-River Germany), 318 ± 17g at the time of surgery, were maintained on a 12-12 h light-dark cycle at 22 ± 0.5°C ambient temperature. They were kept individually in Macrolon cages and provided with food and water *ad libitum*, except food restriction during behavioural training. The experiments were carried out with governmental approval according to local guidelines for the care and use of laboratory animals at the University Hospital Zürich, Switzerland.

Experimental protocol

Two separate experiments were performed. In Experiment 1 (Fig. 4.2.1A), rats were implanted with EEG and electromyogram (EMG) electrodes. Animals were then subjected to focal cerebral ischemia (isch) or sham surgery and assigned to one of the three experimental groups: Bac/isch (n = 6), saline/isch (n = 7) or Bac/sham (n = 4). Baclofen (Sigma-Aldrich, Switzerland; 10 mg/kg) was diluted in saline (0.9% NaCl) to obtain 3 mg/ml working solution. The drug was administered intraperitoneally (i.p.) 24 h after surgery and then twice daily (1 h after light onset and offset) for 10 consecutive days. Sleep was recorded during baseline preceding surgery and on day 2, 6 and 11 after surgery. All animals were decapitated one day after the last injection and their brains were collected for histological analysis.

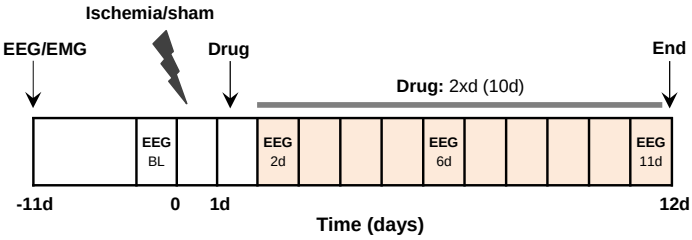
In Experiment 2 (Fig. 4.2.1B), rats were trained in a single pellet reaching task (SPR) for ca. 25 days. During training the preferred paw was identified for every rat.

Cerebral ischemia or sham surgery was performed, when animals reached a stable level of performance. Thereafter, rats were subdivided into three groups Bac/isch (n = 14), saline/isch (n = 14) and Bac/sham (n = 8) and subjected to the same pharmacological protocol as in Experiment 1. Motor function was assessed one day after surgery before the first drug injection and then weekly starting on the day after the end of the drug administration (days 12, 19, 26, 33 and 40 after surgery).

Proliferation marker, 5-bromo-2′-deoxyuridine (BrdU, Sigma Aldrich, St. Louis, MO; 50 mg/kg) (Wojtowicz and Kee, 2006), incorporating into DNA during cell division, was administered for 10 days (2 h after light onset, i.p.), and followed Bac or saline injections. BrdU was diluted in saline (concentration 10 mg/ml).

The anterograde tracer biotinylated dextran amine (BDA, 10%; MW,10,000 Da, Molecular Probes, Eugene, OR, USA; diluted in 0.01M phosphate buffer) (Reiner et al., 2000), used to evaluate axonal sprouting (Carmichael and Chesselet, 2002, Zunzunegui et al., 2011), was micro-injected at two locations into the motor cortex contralateral to the lesion side (stereotaxic coordinates: +/-1 mm AP, 1 mm ML, 3 mm DV from the skull). All rats belonging to the Experiment 2 received a total volume of 1 µl of tracer (0.5 µl of each injection, over 10 minutes) six weeks after surgery. Two weeks later rats were sacrificed and brains were collected for further evaluations.

A. Experiment 1



B. Experiment 2

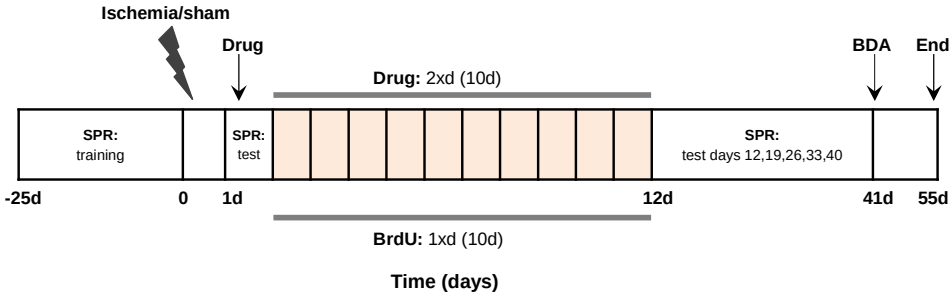


Figure 4.2.1 Design of the experiments
A. In Experiment 1 rats were implanted with EEG/EMG electrodes and 11 days later subjected to ischemia (isch) or sham surgery. 24 h after surgery animals were treated with the drug (10 mg/kg baclofen, Bac, or saline, Sal), and then twice daily for ten days. EEG and EMG were recorded during a 24-h baseline (BL) day and on days 2, 6 and 11 following isch/sham surgery. Three treatment groups were designed: Bac/isch (n = 6), Sal/isch (n = 7) and Bac/sham (n = 4). **B.** In Experiment 2 rats were trained in the single pellet reaching (SPR) task for 3 - 4 weeks preceding isch or sham surgery. 24 h after surgery rats were treated with Bac or Sal, and then twice daily for ten days. All rats received also bromodeoxyuridine (BrdU) injection for ten days. SPR performance was assessed over 3 days preceding surgery (baseline) and on days 1, 12, 19, 26, 33 and 40 following surgery. Forty one day after surgery all rats received microinjection of biotinylated dextran amine (BDA) and were perfused two weeks later. Three treatment groups were designed: Bac/isch (n = 14), Sal/isch (n = 14) and Bac/sham (n = 8).

EEG implantation and recording

Rats were implanted epidurally with EEG and EMG electrodes under deep anaesthesia (2% isoflurane in 30% O_2 and 70% N_2O). Four gold-plated mini-screws were positioned in the skull over the motor cortex of the right and left hemispheres (+/-2 mm to bregma, 2 mm lateral to midline). Electrodes were connected to stainless steel wires and fixed to the skull with dental cement. Two gold wires were inserted bilaterally in the neck muscles for EMG recording. At least 8-10 days were allowed for recovery.

EEG and EMG were sampled at 200 Hz. Signals were amplified, filtered and analog-to-digital converted. Hardware EMBLA and software Somnologica-3 (Medcare Flaga, Iceland) were used. Activity in the 50 Hz band was discarded from the analysis because of power line artifacts. The EEG was subjected to a discrete Fourier transformation yielding power spectra (range: 1–25 Hz; frequency resolution: 0.25-Hz bins; time resolution: consecutive 4-sec epochs; window function: hamming).

Three vigilance states - NREM sleep, REM sleep and wakefulness - were visually scored according to standard criteria at 4-sec epochs (Tobler et al., 1997). In addition to these three conventional vigilance states we introduced a new state, distinct from physiological sleep or wakefulness, which was observed after Bac administration. This state was characterised by atypical behaviour and abnormal hypersynchronous EEG pattern (“drug-induced state”), as described previously (Hodor et al., in review). The state lasted 195.5 ± 7.6 and 197 ± 8.3 min in the Bac/isch group (light and dark phase, respectively), and 103.5 ± 32 and 189.1 ± 11.5 min in the Bac/sham group. Epochs were assigned to a specific vigilance state when more than half of the epoch fulfilled the criteria for that state. Epochs containing EEG artifacts were identified and excluded from

subsequent spectral analysis in both derivations (25% of recording time, most of them (17%) occurred during wakefulness).

Induction of focal cerebral ischemia

Stroke was induced by the 3 vessel occlusion method (3Vo) with permanent occlusion of the distal middle cerebral artery (MCA) and the ipsilateral common carotid artery (CCA), superimposed by temporal occlusion of the contralateral CCA under general anaesthesia with 2% isoflurane (Chen et al., 1986, Zunzunegui et al., 2011). A small piece of the skull overlying the MCA was removed and the dura was retracted. The MCA and its three main branches were occluded by bipolar electrocoagulation. The CCA ipsilateral to the occluded MCA was ligated permanently with a 4-0 silk suture, whereas the contralateral CCA was temporarily occluded for 60 min with an aneurysm clip. Rectal temperature was maintained between 36.5 ± 0.5°C by a warm lamp during the surgery. Sham-operated rats were subjected to the same procedure except for occlusion of the MCA and CCA. Both ischemia and sham surgeries were performed on the hemisphere contralateral to the preferred forelimb assessed by SPR task.

Single pellet reaching (SPR) task

SPR task was used to assess fine motor skills (Gharbawie et al., 2005a). Rats had to use their preferred forelimb to retrieve a food pellet located in a well outside the test chamber (Zunzunegui et al., 2011). Briefly, animals were placed in a clear Plexiglas box (41×27×37 cm) with a vertical slit (1×15 cm) placed in the middle of the front wall, 1 cm above the floor. A 2-cm wide shelf with small wells was mounted in the front of the slit, but outside the box wall. Animals were trained to reach a food pellet (45 mg dustless precision pellet, Bio-Serv, Frenchtown, NJ, USA) placed in the well on the shelf. Rats received daily training sessions consisting of 50 pellets for 3-4 weeks. A pellet was placed in the well on the side contralateral to the preferred paw. A single reaching attempt was permitted. Reaching attempts were classified as successful or failed. In the successful attempt the rat was expected to make a single reach, grasp the pellet from the well, bring it to the mouth and eat it. During the test sessions before and after surgery rats were given 50 pellets and the session ended when rats made 50 attempts or when 15 min elapsed. Success rate was computed as the percentage of successfully obtained pellets out of 50 possible attempts. The baseline (BL) performance was computed as the

average of the three days immediately preceding surgery. Improvement in post-stroke motor performance was computed as a difference in success rate between days 40 and 1 after ischemia.

To increase motivation all animals underwent a food restriction schedule with 20 grams of chow per day during the training weeks and at the days preceding the test sessions. During this time rats were maintained at 95% of their normal body weight.

Tissue collection

Two weeks after BDA administration rats were deeply anaesthetised and perfused transcardially with ice-cold 4% PFA in phosphate buffered saline (PBS). Brains were removed, post-fixed in PFA for 2 h and cryoprotected in 15 and 30% sucrose in the ascending manner. The tissue was stored at -80°C for further evaluations.

Analysis of lesion volume and corpus callosum thickness

To determine lesion size and corpus callosum thickness, 40 μm thick coronal sections were cut with a cryostat at six predefined levels: 2.7 (L1), 1.7 (L2), 0.7 (L3), −0.3 (L4), −1.3 (L5), and −2.3 (L6) mm to bregma (Paxinos and Watson, 2007). Brain sections were then stained with cresyl violet and digitized. Measurements were done with ImageJ (NIH, Bethesda, MD, USA). Brain damage was computed on one section for each level as a difference between intact hemisphere and the non-lesioned area of the ischemic hemisphere. Lesion volume was estimated by multiplying obtained brain damage values by the size of each level (section thickness plus distance between levels). The corpus callosum thickness was measured using coronal sections from L6 level.

Immunohistochemistry

For detection of BDA, free-floating brain sections were incubated overnight with avidin-biotin-peroxidase complex (Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) and revealed with 3,3'-diaminobenzidine (DAB, Sigma) (Zunzunegui et al., 2011). The sections were digitized and the area of BDA labeled axons was quantified by determining the number of pixels above the intensity gray scale threshold using ImageJ (NIH, Bethesda, MD, USA) as previously described (Carmichael and Chesselet, 2002). Briefly, the mean surface area of BDA labeled axons was measured in the primary motor cortex and striatum at the levels L2-L4 of coronal sections. Ratios between ipsilesional

and contralesional cortical surface areas (corresponding to the corticocortical projections) and between ipsilesional striatal and contralesional cortical areas (corresponding to the corticostriatal projections) were used as an index of axonal sprouting. For each rat 2 adjacent sections were averaged on each level (n = 9, n = 10, n = 7 for corticocortical and n = 7, n = 7, n = 6 for corticostriatal in the saline/isch, Bac/isch and Bac/sham group, respectively). Ratios closed to zero indicated a low BDA penetration from the contra- to the ipsilesional side and, therefore, limited axonal sprouting.

Double immunofluorescence staining with the antibody against BrdU together with antibodies against specific cell type marker was used to detect cell proliferation and to assess the type of proliferating cells. Free-floating sections were incubated in 2M HCl for 2 h at room temperature (RT) to denaturate DNA and then washed four times in PBS pH=7.4. After preincubation in blocking solution (0.01M PBS containing 2% appropriate normal sera and 0.3% Triton-X), sections were incubated overnight at +4°C with the rat anti-BrdU antibody (1:200, Abcam, Cambridge, UK) and one of the following antibodies for a specific cell type marker: mouse anti-NeuN (1:200, Millipore, Billerica, MA, USA; marker for neurons), rabbit anti-glial fibrillary acidic protein (GFAP, 1:200, Dako, Carpinteria, CA, USA; marker for astrocytes) or rabbit anti-ionized calcium binding adapter molecule 1 (Iba1, 1:600, Wako Chemicals, Osaka, Japan; marker for microglia). Sections were then incubated for 1.5 h at RT with the fluorophore-conjugated secondary antibodies Alexa Fluor-488 (green; for detection of BrdU) or Cy3 (red; for detection of other markers) (1:200, Jackson Immunoresearch, West Grove, PA, USA) against the appropriate host species of the primary antibodies. Finally, sections were rinsed three times with PBS, mounted on gelatin subbed slides (Southern Biotechnology Association, Inc, USA) and cover-slipped.

Cell counting and microscopy analysis

The number of BrdU positive (+), NeuN+/BrdU+, GFAP+/BrdU+ and Iba1+/BrdU+ cells were quantified in the peri-infarct region of ischemic animals and in the corresponding cortical region of sham animals using the optical fractionator probe (Stereo Investigator version 8.2, MicroBrightField Inc., Williston, VT, USA) at 40x magnification on a fluorescence microscope equipped with a motorized x-y stage (Zeiss Axio Imager Z1, Germany; 20x/0.5 EC Plan-Neofluar objective). The peri-infarct area was outlined on a

10x magnification using the tracing function of Stereo Investigator. Several parameters were then determined on optical fractionator: counting frame (200 x 200 µm; x–y plane), optical dissector height (27-36 µm; z plane), distance between sampling regions (600 µm in x and y-direction) and the grid size. A computer driven motor stage allowed to analyze sections at each of the counting frame location under a 40x magnification. This procedure provided unbiased stereological quantification, because once the region of interest was outlined, sampling sites were evenly and randomly distributed throughout the marked region. Quantification was performed at levels L2 and L3 and averaged on 2 sections per animal (n = 7 per ischemic groups, n = 6 per sham group). The data are presented as the average of cell number per mm^2. All histological and immunohistochemical analyses were determined in a blinded way.

Statistical analysis

Effects of Bac treatment and time on motor performance in the SPR task were evaluated by a repeated measures ANOVA (SAS software, SAS Institute, Cary, NC, USA). Effects of treatment on sleep, neurogenesis, axonal sprouting, corpus callosum thickness and brain damage were evaluated by one-way ANOVA. Post-hoc paired and unpaired *t*-tests, Wilcoxon and Kruskal-Wallis or Tukey-Kramer test for multiple comparisons were performed if the results of the ANOVA reached statistical significance ($p<0.05$). All provided values are means ± SEM. Pearson correlation coefficients were calculated between SPR performance and BDA or BrdU parameters; $p<0.05$ was considered of statistical significance.

RESULTS

Effects of Bac on behaviour, vigilance states and EEG power spectrum

Bac increased the amount of NREM sleep during the dark period on days 2, 6 and 11 after stroke compared to saline administration ($p<0.05$, unpaired *t*-test; Fig. 4.2.2A). We found no changes in the amount of REM sleep after Bac treatment.

EEG power spectra were affected mostly in the hemisphere ipsilateral to the lesion. Stroke led in fact to a significant reduction of EEG power density in NREM sleep in the almost entire frequency range (Fig. 4.2.2B). Thus, in the saline/isch group it was reduced below BL values in the frequencies > 3.75 Hz during both, light and dark phases

on days 2 and 6 after stroke and during light phase on day 11 (Fig. 4.2.2B left). Bac treatment resulted in a partial recovery of power. Hence, a significant reduction of power density in the frequencies > 4.75 Hz was observed only during the light phase on day 2 after stroke (Fig. 4.2.2B right). EEG power was below BL values in the frequencies between 5.75 and 7.75 Hz on days 6 and 11 (only light phase) and above 17 Hz on day 6 (Fig. 4.2.2B right).

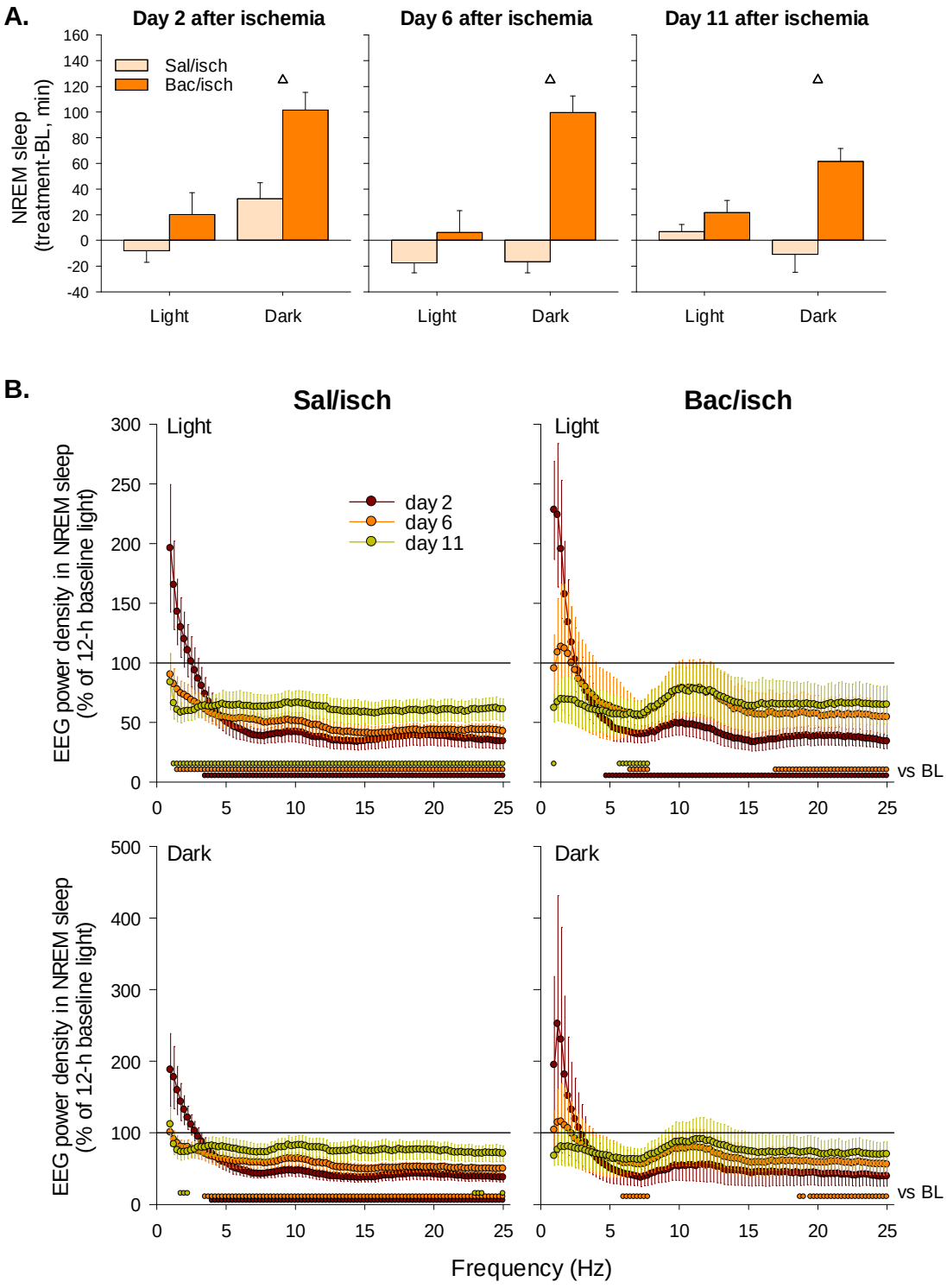


Figure 4.2.2 Effect of baclofen (Bac) or saline (Sal) treatment on NREM sleep amount (minutes) (A) and EEG power density in NREM sleep (B) during the light and dark phase of days 2, 6 and 11 after stroke.

A. Amount of NREM sleep (mean ± SEM) was computed over the 11-h period following drug injection as a difference between treatment and corresponding baseline (BL) level. $^{\Delta}p<0.05$, unpaired *t*-test. **B.** Power in each frequency bin after Bac or Sal treatment was normalised to the corresponding mean 12-h light BL value of the same bin. The curves connect mean values ± SEM during the light and dark phase. Circles indicate differences between the treatment day and BL: $p<0.05$, paired *t*-test.

Effects of Bac on functional recovery

Effects of Bac administration on the recovery of grasping ability were assessed by SPR task. All rats showed similar performance in the task prior to ischemia. The success rate of pellet retrieval was 51.14 ± 2.75 %, 51.36 ± 2.81 % and 45.50 ± 2.74 % in Bac/isch, saline/isch and Bac/sham groups, respectively. Rat performance dropped to zero immediately after stroke in both ischemic groups, but remained stable in the sham-operated animals (Fig. 4.2.3A). Slow spontaneous recovery was observed in the saline/isch group in the course of the following six weeks. However, saline/isch rats never reached the performance level of sham-operated animals ($p<0.005$, Kruskal-Wallis after rANOVA interaction 'group' x 'day' $F_{(12,198)}=7.58$, $p<0.0001$; Fig. 4.2.3A). In contrast, pellet retrieval of the Bac/isch group did not differ significantly from the Bac/sham group starting from day 33 post-stroke. Moreover, ischemic rats treated with Bac had generally a higher success rate than saline-treated animals ($p<0.01$, Tukey-Kramer 'group' after rANOVA factor 'group' $F_{(2,33)}=12.51$, $p<0.0001$; Fig. 4.2.3A).

It has been shown previously that the side of the brain lesion may affect the dynamics of functional recovery (Miklyaeva et al., 1993). Therefore, we investigated the effect of the lesion side on the success rate of pellet retrieval. Bac facilitated motor function recovery only in rats with right hemispheric stroke ($p<0.0001$, Tukey-Kramer 'group' after rANOVA factor 'group' $F_{(2,17)}=9.68$, $p=0.0016$; Fig. 4.2.3B). Improvement in performance was detected already at day 19 after surgery. No such facilitation was observed in rats with left hemispheric stroke (Tukey-Kramer 'group' n.s.; Fig. 4.2.3C). Moreover, both ischemic groups with left hemispheric stroke showed no significant difference from sham-operated animals starting from day 26 after surgery (Fig. 4.2.3C).

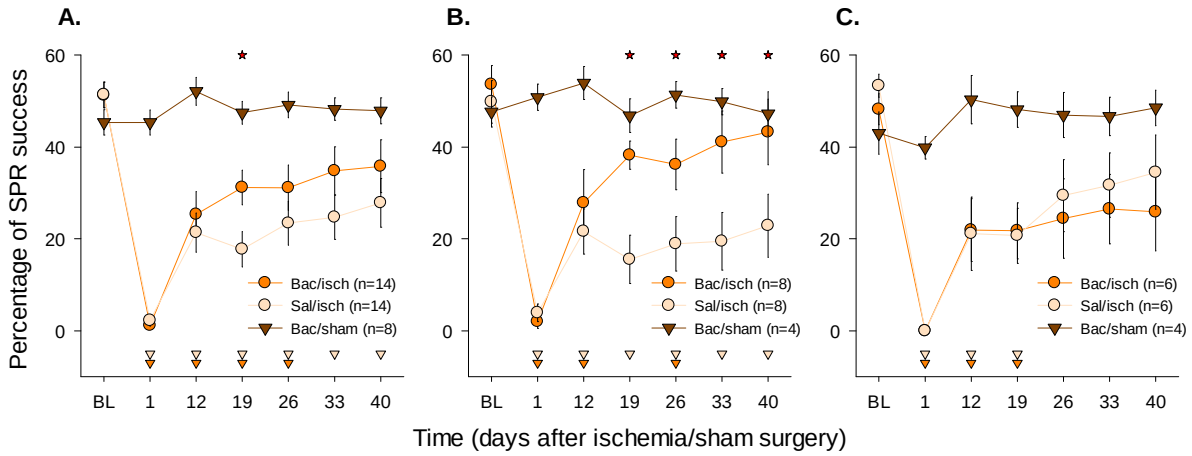


Figure 4.2.3 Effects of baclofen (Bac) or saline (Sal) treatment on motor function recovery in the single pellet reaching (SPR) task following brain ischemia (isch) or sham surgery: all rats pooled **(A)**, rats with lesion in the right **(B)** or left hemisphere **(C)**. The SPR success (number of successful attempts) was expressed as percentage of the total reaching attempts made (mean ± SEM). Triangles indicate difference between Bac/sham and Bac/isch (orange) or Sal/isch (light orange) groups; stars between Bac/isch and Sal/isch group: $p<0.05$, unpaired *t*-test following significant ANOVA.

Effects of Bac on axonal sprouting

Functional recovery can be related to neuroanatomical changes of tracts of fibers originating in the unlesioned cortex (Wiessner et al., 2003). Therefore, we evaluated axonal sprouting from neurons in the contralesional motor cortex towards the ipsilesional hemisphere (cortex and striatum; Fig. 4.2.4). Ratios reflecting corticocortical and corticostriatal projections were closed to zero in the Bac/sham rats, indicating that treatment did not affect axonal sprouting in the control situation (Fig. 4.2.4B). In the saline/isch group only the corticostriatal ratio showed a significant increase compared to sham values ($p<0.005$, Tukey-Kramer after ANOVA factor 'group' $F_{(2.19)}=57.10$, $p<0.0001$; Fig. 4.2.4B). In contrast, Bac treatment in ischemic rats resulted in a 2-fold increase of both ratios compared to either saline/isch or Bac/sham group ($p<0.0001$, Tukey-Kramer after ANOVA factor 'group' $F_{(2.25)}=54.46$ and $F_{(2.19)}=57.10$, $p<0.0001$ for corticocortical and corticostriatal ratios, respectively; Fig. 4.2.4B), indicating pronounced axonal sprouting from contra- to ipsilesional hemisphere. Moreover, ratios reflecting corticocortical and corticostriatal projections correlated positively with the improvement in motor performance ($r=0.53$, $p<0.01$, $n = 26$ and $r=0.47$, $p<0.05$, $n = 20$, respectively).

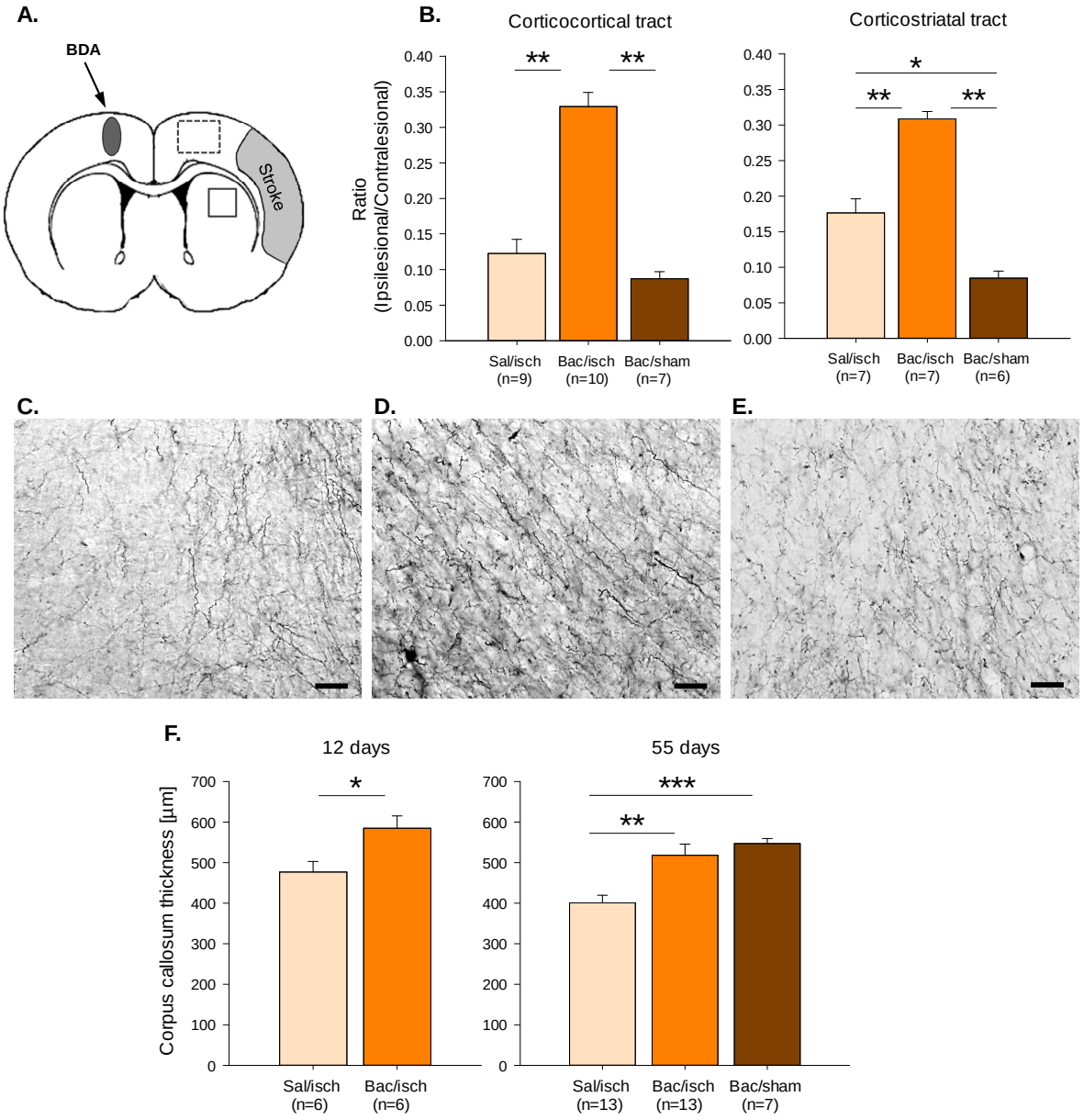


Figure 4.2.4 Effect of baclofen (Bac) or saline (Sal) treatment on axonal sprouting (mean ± SEM) into the lesion-sided motor cortex and striatum following ischemia (isch) or sham surgery.

A. Schematic coronal brain section illustrating site of the lesion and placement of biotinylated dextran amine (BDA) tracer injection. **B.** Quantification of corticocortical (left panel, dashed-box area in A) and corticostriatal (right panel, black-boxed area in A) projections stained with BDA. Differences between groups: *p<0.005, **p<0.0001, Tukey-Kramer following significant ANOVA. **C-E:** High-power magnification photographs from representative Sal/isch **(C)**, Bac/isch **(D)** and Bac/sham **(E)** rats showing BDA traced fibers in the ipsilesional motor cortex (dash-boxed area in A). Scale bars, 50 µm. **F.** Effect of Bac or Sal treatment on stroke-induced corpus callosum atrophy (mean ± SEM) 12 and 55 days after isch or sham surgery . *p<0.05, unpaired *t*-test; ** p<0.005, ***p<0.001, Tukey-Kramer following significant ANOVA.

Effects of Bac on neurogenesis

After stroke the peri-infarct area shows increased neuroplasticity, which allows sensorimotor functions remapping (Dijkhuizen et al., 2003, Cramer, 2008). In order to evaluate the effect of Bac treatment on neurogenesis, we assessed the number of BrdU+ cells in the peri-infarct region eight weeks after ischemia. Consistent with previous reports (Shin et al., 2008, Zunzunegui et al., 2011), we found a prominent BrdU labelling in both ischemic groups in contrast to sham animals, which showed a low level of cell proliferation. Moreover, there was a 60 % increase in the number of BrdU+ cells in the Bac/isch (527 ± 32 cells/mm^2) compared to the saline/isch group (317 ± 24 cells/mm^2; $p<0.0001$, Tukey-Kramer after ANOVA factor 'group' $F_{(2.26)}=59.22$, $p<0.0001$).

To characterise the fate of BrdU+ cells, we examined double immunostaining with different cell-specific markers (NeuN for neurons, GFAP for astrocytes and Iba1 for microglia). Quantification of double labelled cells revealed that Bac administration after ischemia induced a significant increase of all targeted cell types compared to both saline/isch and Bac/sham groups. The number of NeuN+/BrdU+, GFAP+/BrdU+ and Iba1+/BrdU+ cells was significantly above saline/isch values 55 days after stroke ($p<0.0001$, Tukey-Kramer after ANOVA factor 'group' $F_{(2.19)}=70.53$, $F_{(2.19)}=74.89$, and $F_{(2.19)}=18.11$, $p<0.0001$, respectively; Fig. 4.2.5A). In addition, the number of newborn neurons, astrocytes and microglia correlated positively with the improvement in SPR task ($r=0.71$, $p<0.0005$; $r=0.74$, $p<0.0005$; $r=0.57$, $p<0.01$, $n = 20$, respectively).

In all groups the majority of BrdU+ cells co-localized with the neuronal marker NeuN (Fig. 4.2.5B). The percentage of NeuN+/BrdU+ cells in both ischemic groups was significantly higher compared to sham-operated animals ($p<0.001$, Tukey-Kramer after ANOVA factor 'group' $F_{(2.19)}=12.08$, $p<0.05$). No difference was found between the two ischemic groups. Interestingly, the proportion of GFAP+/BrdU+ cells was significantly higher in Bac/isch compared to both saline/isch and Bac/sham groups ($p<0.0001$, Tukey-Kramer after ANOVA factor 'group' $F_{(2.19)}=13.53$, $p<0.05$; Fig. 4.2.5B). Additionally, the proportion of NeuN+/BrdU+ and GFAP+/BrdU+ cells correlated positively with the recovery of post-ischemic SPR success ($r=0.69$, $p<0.001$; $r=0.65$, $p<0.005$; $n = 20$, respectively). The percentage of BrdU+ cells co-expressing Iba1 did not differ between groups.

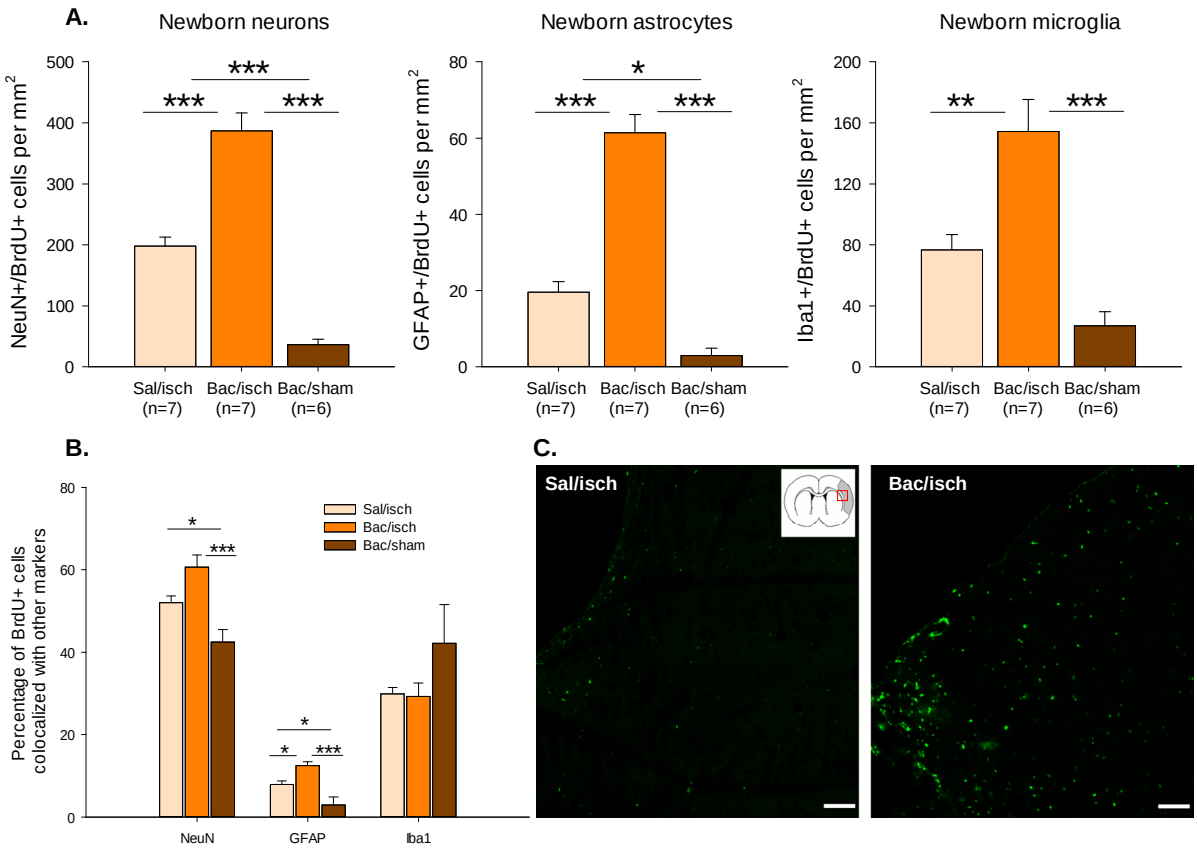


Figure 4.2.5 Effects of baclofen (Bac) or saline (Sal) treatment on cell proliferation in the peri-infarct region 55 days after ischemia (isch) or sham surgery.

A. Number (mean ± SEM) of newborn neurons (NeuN+/BrdU+ cells), astrocytes (GFAP+/BrdU+ cells) and microglia (Iba1+/BrdU+ cells) per mm². **B.** Proportion of BrdU+ cells expressing NeuN, GFAP or Iba1 in the three experimental groups. Differences between groups: *p<0.05, **p<0.005, ***p<0.0001, Tukey-Kramer after significant ANOVA. **C.** Representative confocal images of BrdU-positive cells in the peri-infarct region (red-boxed area on the brain scheme) of Sal/isch and Bac/isch groups. Scale bars, 100 µm.

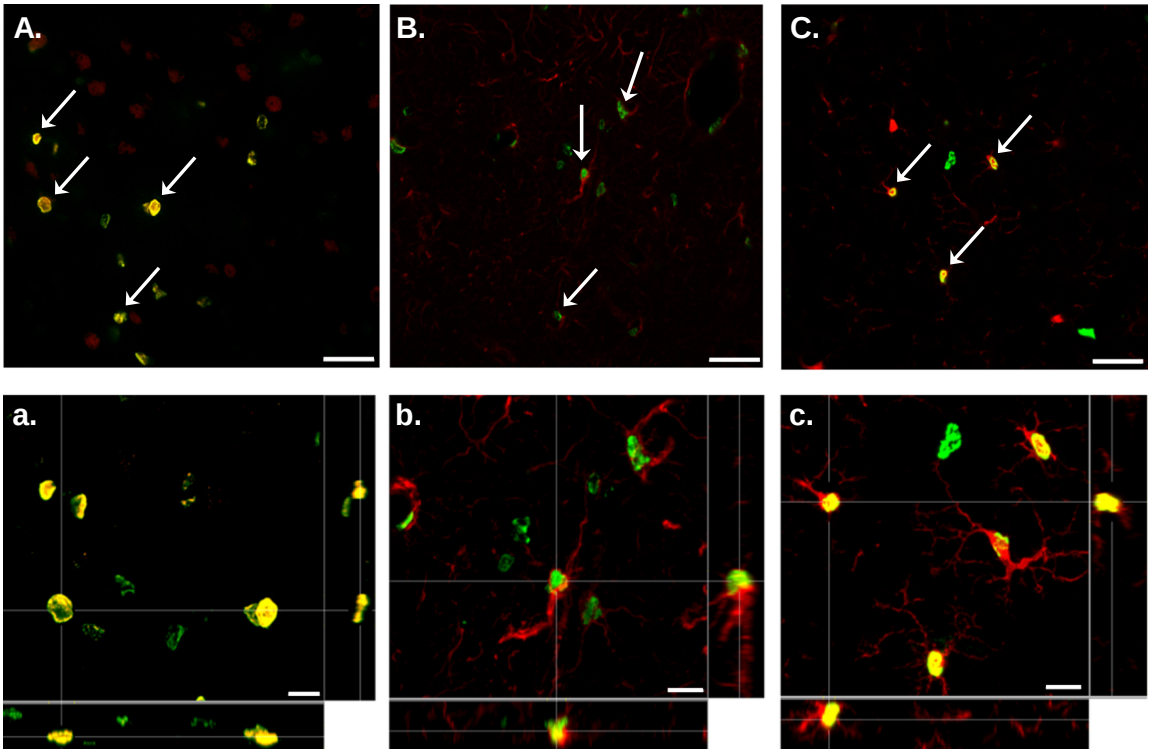


Figure 4.2.6 Phenotype of BrdU-positive cells (representative confocal images). Coronal sections were double labeled (arrows) for BrdU (green) and NeuN **(A)**, GFAP **(B)** or Iba1 **(C)** (red). Scale bars, 30 µm. **a-c:** Orthogonal reconstructions confirming double labeling in x–z (bottom) and y–z (right) planes. Scale bars, 10 µm.

Effects of Bac on lesion volume and corpus callosum thickness

Bac treatment had no effect on the extension of the sensorimotor cortex damage. Lesion size differed between Bac- and saline-treated animals neither 12 (55.5 ± 10.1 vs. 57.5 ± 8.8 mm^3, respectively; n.s.) nor 55 days after ischemia (61.8 ± 11.1 vs. 71 ± 9.6 mm^3, respectively; n.s.). The mean volume of the right hemisphere lesion (66.8 ± 14.9 and 76.4 ± 10.9 mm^3; Bac and saline group, respectively) did not differ significantly from the left hemisphere lesion (55 ± 17.9 and 62.4 ± 18.8 mm^3).

It has been shown that ischemia results in corpus callosum atrophy, which is considered to be an indirect marker of neuronal loss (Yamauchi et al., 2000). To further evaluate the effects of Bac treatment on the ischemic brain, we determined thickness of corpus callosum. Thickness of corpus callosum was reduced in the saline/isch group when compared to the Bac/sham group ($p<001$, Tukey-Kramer after ANOVA factor 'group' $F_{(2,32)}=11.18$, $p=0.0002$, n = 33; Fig. 4.2.4F). Bac treatment of ischemic rats decreased the atrophy of the corpus callosum significantly compared to saline treatment both at 12 ($p<05$, unpaired *t*-test, n = 12; Fig. 4.2.4F) and 55 days after ischemia ($p<005$, Tukey-Kramer after ANOVA factor 'group' $F_{(2,32)}=11.18$, $p=0.0002$, n = 33; Fig. 4.2.4F).

DISCUSSION

In the present study we show for the first time to our best knowledge, that pharmacological sleep promotion with baclofen (Bac) 1) has beneficial impact on long-term functional recovery after stroke in rats, and 2) this improvement in behavioural performance is associated with an enhancement in endogenous brain restorative processes, such as axonal sprouting and neurogenesis.

Sleep has been suggested to be important during the recovery process after stroke. We have previously shown that sleep disruption after cerebral ischemia impairs functional and structural outcomes (Zunzunegui et al., 2011), and that SD before stroke, leading to sleep rebound in the acute phase of ischemia, is instead neuroprotective (Cam et al., 2013).

In this study the main goal was to test the effects of pharmacological sleep promotion wih Bac starting 24 h after stroke onset, in order to avoid the reported neuroprotective effects of this compound (Jackson-Friedman et al., 1997, Zhang et al., 2007).

In accordance with the previous studies in rodents (Baumann et al., 2006, Leemburg, 2011), ischemic lesion resulted in a broad inhibition of the faster part of the EEG spectrum in the lesioned hemisphere. Bac administration following ischemia resulted in a significant increase in NREM sleep amount, particularly during the dark period. Bac given at the beginning of the light phase was less effective probably because of a ceiling effect. Furthermore, Bac led to a recovery of the power in the higher frequencies (above 8 Hz). Noteworthy, recent observations in rats, linked motor function recovery with EEG frequencies > 7 Hz (Leemburg, 2011, Zhang et al., 2013). SWA during NREM sleep has been associated with neuroplasticity processes (Tononi and Cirelli, 2006). Low-frequency synchronous neuronal activity has been suggested to have an important role in the anatomical reorganization and axonal sprouting after brain lesion (Carmichael and Chesselet, 2002). Therefore, the changes in EEG activity observed immediately after Bac injections could play a role in improving the functional outcome.

In the present study cerebral ischemia was induced in the somatosensory cortex, thus leaving the motor cortical areas anatomically intact. Nevertheless, we observed a remarkable drop in the SPR performance after stroke. This effect was expected since motor cortex function was severely disrupted by extensive remodeling processes,

during the recovery period following ischemia even when the area itself is spared by the lesion (Gharbawie et al., 2005b). Spontaneous gradual improvement of motor function was present in the stroke animals injected with saline. However, Bac treatment accelerated performance improvement after stroke.

After Bac treatment, we found increased BDA labeling in both, cortex and striatum, indicating an enhancement in the number of axons and/or increased axonal transport. Axonal sprouting, or the ability of brain to form new connections in areas denervated by the lesion, is a well-known phenomenon (Carmichael and Chesselet, 2002, Wiessner et al., 2003). Ischemic damage of the sensorimotor cortex induced sprouting of axons into the peri-lesion cortex from the homotopic cortex in the contralateral hemisphere (Carmichael and Chesselet, 2002) and into the striatum below the lesion (Napieralski et al., 1996).

Noteworthy, the corpus callosum atrophy was significantly reduced in Bac/isch group, despite the absence of the beneficial effect of Bac on the extent of brain damage. The atrophy of corpus callosum is a sensitive indicator of ischemic neuronal loss, since majority of the neurons projecting into the corpus callosum are highly vulnerable to ischemia pyramidal cells of cortical layers 3 and 4 (Yamauchi et al., 2000). Because corpus callosum is the conduit for the inter-hemispheric communication, its thickness might be a relevant factor influencing recovery of function after stroke. Our finding that Bac administration significantly reduced stroke-induced corpus callosum atrophy supplements the data showing a Bac-related boost in axonal sprouting.

Repeated administration of Bac also boosted neurogenesis in the ischemic brain. We found an increase in the number of newborn cells. Several reports indicated that ischemic injury induces increased cell proliferation (Zhang et al., 2001b, Shin et al., 2008), associated with migration of newborn cells to the lesion sites (Arvidsson et al., 2002, Parent et al., 2002, Jin et al., 2003). However, only a small fraction of newborn cells display a long-term survival. Therefore, enhancement of endogenous neurogenesis, primarily by improving survival of newborn cells, would be a plausible strategy for restorative therapies. In our study BrdU was injected for 10 days after induction of ischemia, parallel with drug injection, and examined six weeks later. After extended periods, very few BrdU+ cells were found in the ipsilesional cortex, striatum and subventricular zone. In contrast, extensive BrdU staining was noted in the peri-infarct region in both ischemic groups. Peri-infarct area is critical for rehabilitation, it shows

intensified neuroplasticity, allowing remapping of sensorimotor function (Dijkhuizen et al., 2003, Cramer, 2008). Bac-treated rats had almost 2-fold increase in BrdU+ cells 55 days after stroke onset. Hence Bac might not only increase cell proliferation, but also prolong survival of newborn cells and, therefore, enhance endogenous neurogenesis.

Although the majority of newly formed cells found in the peri-infarct region expressed the neuronal marker NeuN, we also observed an increase in the number of proliferating cells expressing glial markers, GFAP (astrocytes) and Iba1 (microglia) after Bac treatment. Interestingly, the percentage of cells that differentiated into astrocytes was significantly higher in Bac/isch group, indicating that Bac might also affect differentiation of newborn cells. Glial cell activation has been demonstrated to accompany cerebral ischemia. However, there is a disagreement whether such gliosis is neuroprotective or harmful (Nedergaard and Dirnagl, 2005). Recent experimental evidence indicates that astrocytes and microglia play a dual role in tissue repair and reorganization (Swanson et al., 2004, Pekny and Nilsson, 2005, Ekdahl et al., 2009). Attenuation of reactive gliosis led to enlarged infarct volume, (Li et al., 2008), while enhanced survival of astrocytes by melatonin treatment after cerebral ischemia, conferred protection to the injured brain (Borlongan et al., 2000). Furthermore, defective microglial activation was associated with significant increase in the size of ischemic lesion and a 2-fold increase in the number of apoptotic neurons (Lalancette-Hebert et al., 2012). The results of the present study are in accordance with the emerging view that glial cells are active participants in the maintenance of a functional central nervous system and play an important role in the recovery from the brain lesion.

We observed that the increase in axonal sprouting and neurogenesis in Bac treated animals was positively correlated with the improvement of functional recovery in the SPR task during the 40 days period after stroke. Several studies have previously suggested an association between neuronal plasticity and neurological recovery. In experimental stroke, enhancement of axonal sprouting improves functional outcome after brain damage (Kawamata et al., 1997, Stroemer et al., 1998, Carmichael, 2008). In addition, disruption of neurogenesis impedes functional recovery after stroke, whereas treatment strategies aimed at augmenting neurogenesis are associated with functional improvement (Zhang et al., 2001a, Raber et al., 2004). We suggest that Bac treatment, most likely by promoting NREM sleep, enhanced endogenous mechanisms underlying neuronal plasticity and, therefore, improved functional recovery.

One intriguing finding of this study was that facilitation of functional recovery by Bac depended on the location of the lesion. Right hemisphere lesioned rats treated with Bac recovered faster, although the stroke extension/volume was similar on both sides. Brain asymmetry in motor and other functions was found in humans and rodents (Habib, 1989, Sullivan and Gratton, 1999). The biochemical and anatomical origin of lateralised behavioural responses remains however poorly understood.

In summary, we have shown that an increase of NREM sleep induced by the delayed administration of baclofen promotes neuroplasticity and functional outcome in a rat model of stroke. Further studies are needed to understand the mechanisms responsible for these sleep-associated favourable effects.

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5. GENERAL DISCUSSION

Stroke is often referred to as the 'silent killer' because, unlike heart attack, it strikes without warning. Although acute ischemic stroke is a major problem, due to the continuous failure of compounds developed for its treatment, an effective therapy is lacking. Still, there is a great hope in this field. Recent data indicate that it might be possible to successfully treat a significant portion of stroke patients by promoting endogenous neuroplasticity. A big body of evidence demonstrates a remarkable ability of adult brain to reorganize and remap functional networks leading to recovery from stroke damage. One of the biggest experimental challenges in this field is to unequivocally link specific circuit changes with the improvement in behavioural outcomes observed during the recovery period. Thus, generation of animal models of ischemic brain lesion and investigation of new genetic tools, which could be applied, are currently of major interest. Mechanisms in which activity can scale down synaptic strength (that is, mechanisms that can induce homeostatic plasticity), such as sleep, can represent a big promise in humans.

Clinical data have indicated sleep-wake disturbances as risk factors for the development of many neurological diseases, including ischemic stroke. Moreover, sleep disorders frequently occur in stroke patients and are related to worse recovery (Bassetti and Hermann, 2011). As previously introduced, experimental data indicated that sleep disruption after stroke aggravates brain damage and impairs recovery. If this conclusion is true, it could be assumed that an increase in sleep would have favourable effects on stroke outcome. Although sleep appears to facilitate neuronal plasticity in the healthy brain, its role in modulating the restorative process after brain injury is almost unknown. In these studies we manipulated sleep by means of a pharmacological approach in a rat model of focal cerebral ischemia. The results obtained in this thesis, not only support the close relationship between brain plasticity, stroke outcome and sleep, but also identify sleep as a tool to improve brain recovery from neuronal damage. This could lead to development of a possible sleep-modulating treatment to improve stroke outcomes.

5.1 Bac and GHB effects on sleep

In the first study, we aimed to check whether and how Bac and GHB affected sleep in healthy rats.

There is a big body of evidence that GABA plays a major role in sleep regulation (Gottesmann, 2002). A prominent role of $GABA_A$ receptors in sleep, in particular the pharmacological management of disturbed sleep has been established (Winsky-Sommerer, 2009). $GABA_A$ receptor agonists and different compounds interacting with the $GABA_A$ receptor complex, such as barbiturates and benzodiazepines, can interfere with the sleep/wake cycle (Sieghart, 1995). On the other side, there is still little information about the importance of $GABA_B$ receptors in sleep modulation and EEG changes. They have been reported to be important in several conditions, such as epilepsy (Schuler et al., 2001), anxiety and depression (Mombereau et al., 2004), nociception (Zarrindast et al., 2000), memory (Levin et al., 2004), addiction (Cruz et al., 2004, Filip and Frankowska, 2008) and potentially sleep (Juhasz et al., 1994, Ulloor et al., 2004). Both, Bac and GHB are ligands of $GABA_B$ receptors. Of importance, both of the drugs are currently in clinical use, Bac to treat spasticity (Albright et al., 1991, Paisley et al., 2002, Bensmail et al., 2006), while GHB sleep disturbances associated with narcolepsy (Billiard et al., 2006).

The main findings of this investigation were that in rats, Bac and GHB had a strong effect on behaviour, vigilance states and EEG pattern. Most importantly and relevant for our consecutive project, Bac but not GHB, had sleep-promoting properties. In addition, our study was the first to evaluate the effects of Bac and GHB given at two circadian time points, which are associated with different sleep pressure. We found clear differences in the effect on sleep of both substances regarding light-dark cycle. Drugs given at the beginning of the dark period, coinciding with the daily activity phase of rats, revealed more pronounced effects on sleep. Sleep-wake behaviour of rats during the dark period is often used as a physiological model for insomnia in humans and is more sensitive to the hypnotic effects of drugs (Lancel and Langebartels, 2000). Hence, with this study we highlight also the importance of considering the changes in endogenous sleep and the different drug's kinetics according to the circadian phase, while applying different agents in experimental and therapeutic conditions. Furthermore, changes in EEG power spectra during waking, NREM and REM sleep observed in our study might be

related to the complex mechanisms, engaging multiple mutually cooperating neurotransmitter systems. Presently, there is a lot of effort to create drugs acting through GABA receptors in order to promote physiological sleep for therapeutic purposes. Further elaborate research is needed to better understand drug mechanism of action and adverse effects on physiology.

5.2 Sleep promotion and stroke outcome

While sleep disruption in the acute phase after stroke has been shown to have detrimental effects in both humans and animals, the effect of sleep promotion remains unclear. To gain insight into this question we promoted sleep with Bac, starting at 24 h after stroke. In this study we showed that repeated treatment with Bac after focal cerebral ischemia in rats improved long-term functional recovery accompanied by enhanced endogenous brain repairing mechanisms, such as axonal sprouting and neurogenesis. These beneficial effects of Bac on stroke outcome might be mediated by sleep.

Bac, sleep and neuroplasticity

We propose that the positive effects of Bac on axonal sprouting and neurogenesis after cerebral ischemia were related to sleep-induced mechanisms. Axonal sprouting after stroke is regulated by various neuroplasticity-related genes, including growth-promoting and growth-inhibiting molecules (Carmichael et al., 2005). Many of these genes and proteins associated with neuroplasticity have been shown to be modulated by sleep (Cirelli et al., 2004, Basheer et al., 2005). Previous studies showed that sleep deprivation after stroke induced an increase in the expression of neurocan (the main growth-inhibiting molecule to axonal sprouting) (Gao et al., 2010), while GHB decreased it (Gao et al., 2008). Therefore, changes in sleep may affect molecules crucial for post-stroke axonal sprouting. Furthermore, Carmichael et al. demonstrated the strong correlation between axonal sprouting and the periodic synchronized neuronal activity, the same type of activity that is known to be important during development of axonal projections (Carmichael and Chesselet, 2002). They suggested that this rhythmic activity is an initial trigger critical for induction and maintenance of axonal sprouting after

ischemic lesion. In the present study, Bac, besides its effects on sleep, also induced a transient electrophysiological hypersynchronous pattern during the sub-anaesthetic state, which could play a role in axonal growth and functional outcome.

Neurogenesis has been shown to be reduced after several days of sleep deprivation or sleep disruption in healthy rats (Guzman-Marin et al., 2005, Meerlo et al., 2009). Consistent with these results, our previous studies demonstrated that rats subjected to sleep deprivation during the acute and sub-acute phase of ischemic stroke revealed a substantial reduction in the number of proliferating cells (Zunzunegui et al., 2011). These findings imply that mechanisms associated with sleep may promote production of new cells. In other words: if on the one side sleep deprivation reduces neurogenesis, would sleep promotion facilitate it? At present, there is no direct proof that sleep promotes production of new cells directly, however it may be essential for normal functioning of other processes that regulate neurogenesis. In this study, several mechanisms could account for the increase in neurogenesis after stroke. Neurogenesis is regulated and affected by a wide variety of trophic factors, cytokines, hormones, neuromodulators and neurotransmitters (Abrous et al., 2005, Hagg, 2005) that are also altered by sleep loss. Furthermore, sleep has been suggested to be necessary for the anabolic process underlying cell proliferation (Guzman-Marin et al., 2005). The release of anabolic hormones is modulated by sleep, e.g. the release of growth hormone (GH) is associated with SWA in humans (Parker et al., 1969) and rats (Kimura and Tsai, 1984). Sustained sleep deprivation in rats reduced levels of GH and its mediator, insulin-like growth factor (IGF)-1 (Everson and Crowley, 2004), known to promote neurogenesis (Aberg et al., 2000). Several studies indicated that overall brain protein synthesis rate required for cell proliferation is increased in NREM sleep (particularly deep NREM sleep) in primates (Nakanishi et al., 1997) and rats (Ramm and Smith, 1990), compared with waking or REM sleep. Here we show that Bac resulted in prominent increase of NREM sleep amount after stroke, implying facilitating role of sleep in neuroplastic changes. In this frame of reference, our results suggest that Bac-stimulated cell proliferation induced by stroke could have been mediated by sleep-related mechanisms.

In addition to the increase in the number of newborn neurons, Bac enhanced also the amount of proliferating astrocytes and microglia. There is increasing evidence suggesting that glial cells not only play a supportive role for neuronal activity but also have an essential roles in brain plasticity (Todd et al., 2006, Pekny et al., 2007, Ota et al.,

2013, Welberg, 2014). Astrocytes are responsible for regulating the synaptic environment and for maintaining appropriate levels of neurotransmitters and neurotrophins. They influence neuronal signalling, the number of synapses and play a role in non-synaptic transmission (Vernadakis, 1996, Chvatal and Sykova, 2000, Ullian et al., 2001). Microglia, beyond its role as the brain's resident immune cells, have various different functions including playing a critical role during brain development (Derecki and Kipnis, 2013, Zhan et al., 2014). The role of glial cells in neuronal death has become a major research interest during last years. Although a lot of controversy exists about function of glial cells in cerebral ischemia, a solid evidence indicates that they have an active and protective role in the pathophysiology of ischemic injury (Borlongan et al., 2000, Nedergaard and Dirnagl, 2005, Lalancette-Hebert et al., 2007, Lalancette-Hebert et al., 2012). The results of our study support the notion that glial cells are equally active participants as neurons in the maintenance of a functional central nervous system and are closely involved in the brain injury. Therefore, modulation of glial cells activity, aside neurons, may be a promising future target for therapeutic interventions promoting regeneration and plasticity in the brain after various injuries.

Asymmetric effect of lesion on recovery

One of the most intriguing findings of our study was the asymmetric effect of lesion on the degree of functional recovery. The detailed data analysis demonstrated that facilitation of functional recovery by Bac depended on the location of brain lesion. Although cerebral functional asymmetry in animals is still an underappreciated phenomenon, it is not anymore considered as an unique feature of the human brain - the leading concept for many years (Levy, 1977). Brain asymmetry has been extensively investigated in humans and rodents (Habib, 1989, Sullivan and Gratton, 1999), however the biochemical and anatomical origin of lateralised behavioural response has not been precisely understood yet.

There are several mechanisms that could account for the effects observed in the present study. One of the possible explanations is that the course of spontaneous recovery has been reported to be worse after lesions in the right, compared to the lesions in the left hemisphere (Miklyaeva et al., 1993). The reported behavioural asymmetry after stroke in our study is consistent with these findings. Hence, the difference between Bac and saline-treated animals after the right-side injury could

result from the poorer spontaneous recovery, allowing the drug to reveal its efficacy. It is important to mention here that the size of the damage could not be the reason of the observed asymmetry, because the mean volume of the right and left hemispheric lesions were not different. Another possible explanation could be that this lateralised recovery promoted by Bac was the consequence of neuroanatomical or chemical asymmetries in the brain. It was shown that only right cortical or subcortical lesions led to the lateralised behavioural response and this effect was related to biochemical changes generated by the lesion (Robinson and Coyle, 1980, Robinson and Justice, 1986, Starkstein et al., 1988). Numerous studies demonstrated left/right hemispheric asymmetries in the mesocortical dopamine (DA) projections (Slopsema et al., 1982, Carlson et al., 1993), which originate in the ventral tegmental area (VTA) and provide modulatory input to the frontal cortex. In addition, differential sensitivity of the two hemispheres to neurotoxic destruction of noradrenergic (NE) terminals was reported (Robinson and Justice, 1986). Furthermore, asymmetry of the sympathetic nervous system has been shown to be partially involved in the lateralised immune responses (Neveu, 1992). Rats submitted to the right hemisphere lesion had greater sympathetic consequences (e.g. increase in NE plasma level) (Hachinski et al., 1992) and higher immune reactivity (Renoux et al., 1983) than the left-lesioned rats. Interestingly, Bac is known to affect both DA neurons in VTA (Cruz et al., 2004) and NE neurons in locus coeruleus (LC) (Osmanovic and Shefner, 1988). In addition, recent evidence has suggested its anti-inflammatory role (Duthey et al., 2010). Therefore, if DA and NA activity level is changed only after right-side lesion, and Bac is known to affect both DA in VTA (Cruz et al., 2004) and NA in LC (Osmanovic and Shefner, 1988) , it might be that the pronounced effect of Bac in right-lesioned rats is caused by normalization of the balance disturbed by stroke. However, the mechanisms underlying the physiological cortico-subcortical and autonomic interactions dependent on the side of the lesion, remains to be elucidated.

Identification of lateralised behavioural and biochemical response to the brain injury in rats may have important clinical implications. The evidence that patients with damage in the left frontal brain area exhibit symptoms of major depression and catastrophic reactions more frequently, while patients with the right hemispheric injury have greater incidence of inappropriate cheerfulness and apathy, may be explained as an

emotional manifestation of differential biochemical response of the human brain to injury (Robinson et al., 1984).

Concomitantly with asymmetry in functional improvement, we noted also lateralization in axonal sprouting and neurogenesis. Thus, ischemic animals administered with saline, revealed greater corticocortical projections and the number of newborn neurons and astrocytes, after lesion in the left hemisphere relative to these in the right hemisphere (data not shown). Consequently, the endogenous neuroplasticity was decreased in the right-lesioned rats, whose course of the spontaneous recovery was worse. Although the sample size was too small to conduct appropriate statistical analysis, it would be another proof of association between brain plasticity and neurological recovery. Nevertheless, future work should investigate this enthralling question more systematically.

5.3 Outlook

In summary, this thesis provides an excellent basis to further investigate the involvement of sleep in the process of stroke recovery. Based on these results we suggest that sleep promotion after stroke leads to the improvement in stroke outcome. We propose that sleep enhancement by Bac leads to neuroplastic changes, that in turn trigger functional recovery. However, future work needs to prove this hypothesis by identifying specific cellular mechanisms. One way to do so would be to carry out further experiments characterising sleep-mediated pathways involved in stroke pathophysiology and molecular mechanisms that underlie brain repair. In this context, screening gene expression at a large scale would be necessary to pursue the investigation of molecular candidates that mediate the sleep-mediated changes in brain restorative processes.

In the present study we evaluated only Bac as an effective sleep-promoting drug. That was based on the results from the first experiments, which did not reveal sleep-promoting effects of another tested drug, GHB. However, it could be that GHB injected repeatedly, for longer time period, with higher dose would also show positive action. Therefore, it would be appealing to evaluate whether GHB and/or other sleep-promoting drugs, such as for example tiagabine, GABA uptake inhibitor or THIP (4,5,6,7-

Tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol), $GABA_A$ receptor agonist, could have similar effects.

In the study presented here the EEG recordings were performed only until the day 12th after injury, i.e. the last day of the drugs' administration. This duration could have been too short to observe any big changes in the EEG spectral power. It would be interesting to evaluate EEG parameters at a later phase during recovery process, after the end of the drug treatment period. It could be that possible alterations in EEG spectra occurring during this later phase, after drug treatment, have an essential role for brain plasticity. Moreover, further investigation of the role of EEG activity in functional recovery would require a finer array of electrodes allowing direct measurements of synchrony and functional connectivity within the motor cortex. Next, we only evaluated spectral power during NREM sleep. Since other vigilance states may also be affected by Bac treatment and important for recovery process, it would be important to analyse the waking and REM sleep spectra as well.

These findings have a potentially important impact on our understanding of brain restorative processes after damage and deserve further investigation at the molecular, cellular and systemic level. Specifying the role of sleep in brain repair mechanisms will undoubtedly pose a particular challenge for future research.

6. LIST OF ABBREVIATIONS

AMPA,	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Bac,	baclofen
BDA,	biotinylated dextran amine
BDNF,	brain-derived neurotrophic factor
BL,	baseline
BrdU,	5-bromo-2′-deoxyuridine
CCA,	common carotid artery
DA,	dopamine
EEG,	electroencephalogram
EMG,	electromyogram
GABA,	gamma-aminobutyric acid
GH,	growth hormone
GHB,	gamma-hydroxybutyric acid
IGF-1,	insulin-like growth factor 1
i.p.,	intraperitoneal
isch,	ischemia
LC,	locus coeruleus
LTD,	long-term depression
LTP,	long-term potentiation
MCA,	middle cerebral artery
MRN,	median raphe nucleus
NE,	noradrenaline
NREM,	non-rapid eye movement
PBS,	phosphate buffered saline
PFA,	paraformaldehyde
REM,	rapid eye movement
rt-PA,	recombinant tissue plasminogen activator
SD,	sleep deprivation
SDis,	sleep disturbances
SPR,	single pellet reaching
SWA,	slow-wave activity
SWS,	slow-wave sleep
tDCS,	transcranial direct current stimulation
THIP,	4,5,6,7-Tetrahydroisoxazolo[4,5-*c*]pyridin-3-ol
TMS,	transcranial magnetic stimulation
VTA,	ventral tegmental area

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> ***(Jacques Cousteau)***

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obrigado
Dank U
Merci
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Grazie
Thank you
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Gracias
Dziękuję
Děkuji
danke
Kiitos

9. CURRICULUM VITAE

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Education

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Thesis: "Effects of sleep modulation on stroke recovery in rats".
Supervisor: Prof. Claudio L. Bassetti

2002 – 2007 **Master of Science in Biology**, specialization: Neurobiology
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Jagiellonian University, Cracow, Poland

Thesis: "Extracellular recordings of the spontaneous electrical activity of the rat superior colliculus."
Supervisor: Prof. Marian H. Lewandowski

1998 - 2002 **Secondary School No 5 in Cracow, Poland**
General profile with extended programme of Spanish language.
Secondary School Certificate with distinction (2002).

1990 - 2003 **Music education (violoncello and piano classes)**
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Scientific experience

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Laboratory techniques:
neurosurgeries (ischemic stroke, EEG implantation, tracer injections), EEG recordings, behavioural tests, histology, immunohistochemistry, western blot, microscopy

2007 – 2008 **University of Helsinki, Finland**
CIMO Fellowship in the Laboratory of Neuropharmacology
Subject: Persistent action of drugs of abuse on dopamine neurons.

Laboratory techniques:
Whole-cell patch-clamp in the slice-method.

2005 – 2007 **Jagiellonian University, Cracow**, **Poland**
Scientific research in the Laboratory of Neurophysiology and Chronobiology

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List of Publications

1. **Effects of baclofen and gamma-hydroxybutyrate on behaviour, EEG activity and sleep in rats.**
   Sleep 2014, submitted.
   Hodor A, Palchykova S, Gao B, Bassetti CL.

2. **Baclofen facilitates sleep, neuroplasticity and recovery after stroke in rats.**
   J Clin Invest. 2014, submitted.
   Hodor A, Palchykova S, Baracchi F, Noain D, Bassetti CL.

3. **Sleep disturbance impairs stroke recovery in the rat.**
   Sleep 2011 Sep 1;34(9):1261-9.
   Zunzunegui C, Gao B, Cam E, **Hodor A**, Bassetti CL.

4. **Sleep deprivation before stroke is neuroprotective: A pre-ischemic conditioning related to sleep rebound.**
   Exp. Neurol 2013 Sep 247:673-9.
   Cam E, Gao B, Imbach L, **Hodor A**, Bassetti CL.

Presentations

Oral presentations

- Sleep promotion with baclofen improves functional recovery and promotes neuroplasticity after stroke in rats.
  BENESCO (Bern Network Epilepsy Sleep Consciousness) Winter Research Meeting 2014
  Adelboden, 13-15.03.2014

- Effect of baclofen on sleep and stroke in rats.
  Sleep Research Network Bern Meeting
  Adelboden, 7-9.03.2013

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal cerebral ischemia.
  Current and Future Perspectives in Sleep and Circadian Rhythms Research, School of advanced studies
  Cluj-Napoca/Romania, 31.10-3.11.2012

- Sleep-promoting substances and stroke recovery.
  ESRS - EU «Marie Curie » - PENS Training Programme, Training in Sleep Research and Sleep Medicine, Final Symposium
  Kloster Seeon/Germany, 2-6.07.2010

Poster presentations

- Sleep promotion with baclofen improves functional recovery and promotes neuroplasticity after stroke in rats.
  Joint Congress of European Neurology
  Istanbul/Turkey, 31.05-3.06.2014

- Baclofen facilitates sleep, neuroplasticity and functional recovery after stroke in rats.
  Joint Annual Meeting of Swiss Headache Society and Swiss Society for Sleep Research, Sleep Medicine and Chronobiology (SSSSC)
  Luzern, 15-16.05.2014

- Delayed repeated treatment with baclofen promotes neuronal plasticity and functional recovery after stroke in rats.
  Joint Meeting of Swiss Society for Neuroscience and Clinical Neuroscience Bern
  Bern, 24-25.01.2014

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal cerebral ischemia.
  43rd Annual Meeting of the Society for Neuroscience
  San Diego/USA, 9-13.11.2013

- Sleep and motor function after GHB and baclofen administration in healthy rats and rats with focal cerebral ischemia.
  Day of Clinical Research 2013
  Bern, 06.11.2013

- Delayed repeated treatment with baclofen promotes neuronal plasticity and functional recovery after stroke in rats.
  Zürich Neuroscience Center (ZNZ) Symposium 2013
  Zürich, 13.09.2013

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal cerebral ischemia.
  FENS-IBRO School "From Sleep Science to Sleep Medicine"
  Kazbegi (Tbilisi)/Georgia, 18 -23.07.2013

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal cerebral ischemia.
  23rd Meeting of the European Neurological Society
  Barcelona/Spain, 8-11.06.2013

- Sleep and motor function after GHB and baclofen administration in healthy rats and rats with focal cerebral ischemia.
  2nd SFCNS Congress of the Swiss Federation of Clinical Neuro-Societies
  Montreux, 5-7.06.2013

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal cerebral ischemia.
  9th Annual Meeting of the Swiss Stem Cell Network (SSCN) 2013
  Bern, 8.02.2013

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal cerebral ischemia.
  8th Clinical Neuroscience Meeting 2012
  Bern, 4.12.2012

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal cerebral ischemia.
  Day of Clinical Research 2012
  Bern, 14.11.2012

- Sleep and motor function after GHB and baclofen administration in healthy rats and rats with focal cerebral ischemia.
  21th Congress of the European Sleep Research Society
  Paris/France, 4-8.09.2012

- GHB, baclofen and their effects on physiology and behaviour in healthy rats and rats with focal cerebral ischemia.
  Joint Symposium of the Zürich Neuroscience Center (ZNZ) and the NCCR "Neural Plasticity and Repair"
  Zürich, 14-15.06.2012

- Effects of GHB and baclofen on sleep and motor function in healthy rats and rats with focal ischemia.
  Annual Meeting of the Swiss Society for Neuroscience 2012
  Zürich, 3.02.2012